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# CONTENTS

ORIGINAL PAPERS

Comparison of some methods for estimating vegetation periods in maize*
*C. L. Marton, L. Kálmán, T. Árendás, P. Bónis* and *D. Szieberth* ..... 1

Effect of increased UV-B radiation on the anthocyanin content of maize (*Zea mays* L.) leaves*
*J. Pintér, E. Kósa, G. Hadi, Z. Hegyi, T. Spitkó, Z. Tóth, Z. Szigeti, E. Páldi* and *L. C. Marton* ..... 7

Participatory sorghum varietal evaluation and selection in Pakistan*
*S. R. Chughtai, J. Fateh, M. H. Munawwar* and *M. Hussain* ..... 19

Maize population hybrids: Successful genetic resources for breeding programs and potential alternatives to single-cross hybrids*
*M. J. Carena* ..... 27

Quality responses of winter wheat cultivars to nitrogen and fungicide applications in Croatia
*B. Varga, Z. Svečnjak, Z. Jurković* and *M. Pospišil* ..... 37

Competition, light quality and seedling growth of Russian wildrye grass *(Psathyrostachys juncea)*
*P. G. Jefferson* and *R. Muri* ..... 49

Effect of 1-MCP treatment on apple biochemical content and physiological disorders
*U. Moor, K. Karp, P. Põldma* and *M. Starast* ..... 61

Pyrophosphate:fructose 6-phosphate 1-phosphotransferase is involved in the mobilization of sugar reserves in the taproots of cold- and drought-stressed carrot plants
*G. Kovács, S. Sorvari, P. Scott* and *O. Toldi* ..... 71

Effect of reduced tillage on quality traits of soybean [*Glycine max* (L.) Merr.]
*M. Sabo, D. Jug* and *I. Jug* ..... 83

The papers indicated by an asterisk were presented orally at the 20th International Conference of the EUCARPIA Maize and Sorghum Section, held in Budapest, Hungary on 20–24 June, 2006.

Comparison of different methods used in calculating the effect of stripe rust on wheat grain yields
*N. Bolat* and *F. Altay* ..... 89

Quality of maize (*Zea mays* L.) kernels as affected by the NP supplies of the soil
*Z. Izsáki* ..... 99

Effects of acid treatment and composting on bones used as fertilizer
*D. Kovács, G. Kardos* and *G. Füleky* ..... 115

SHORT COMMUNICATION

Correlation analysis of yields and related physiological variables in twelve generations of durum wheat
*S. N. Sharma* and *Y. Sharma* ..... 125

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# COMPARISON OF SOME METHODS FOR ESTIMATING VEGETATION PERIODS IN MAIZE

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A new method has been elaborated to estimate the length of the vegetation period of new maize hybrids. According to this method, the length of the vegetation period is expressed by the FAO number, calculated from the following traits:

1. 50% silking
2. Grain moisture when the average grain moisture of maturity group standards is 25%
3. Grain moisture when the average grain moisture of maturity group standards is 20%
4. Grain moisture at harvest.

The standards of the neighbouring maturity groups are also included in each experiment.

The results obtained with this method were compared to the heat unit (GDD) method and to other methods of calculating FAO numbers. The new method has several advantages over previous techniques based on moisture content alone: the fluctuation of the estimated FAO number over locations and years decreased, as did the significant differences between the calculated FAO numbers; the reliability and precision of the new FAO number is less dependent on the date of harvest (moisture content); and the FAO numbers calculated with the new method are in the closest correlation with the heat unit estimates.

**Key words:** maize, hybrid, maturity group, drying down

## Introduction

In recent years the difference in grain moisture content between hybrids in the early (FAO 200) and late (FAO 500) maturity groups has tended to decrease in the Carpathian Basin. In the 1980s the mean grain moisture at harvest was 21.77% for FAO 200 hybrids and 28.23% for FAO 500 hybrids, a difference of 6.46%. In the 1990s these figures were 20.1% for the earliest group and 24.43% for the latest group, a difference of 4.33% (Marton et al., 1999). This meant that a difference in grain moisture of 1% represented a difference of more than 60 in

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the FAO number (Marton et al., 2002), though the extreme values recorded in different experiments and years covered a very wide range, from 17–189 FAO/1% grain moisture (Marton et al., 2001). On the German system of calculating FAO numbers, a 1% difference in grain moisture content was equivalent to 10 FAO numbers (Miltner and Rath, 1998).

To solve the problem caused by late harvesting and the slight difference in grain moisture between the hybrids a new method was evolved (Szieberth, 2000; Marton et al., 2001) which used four traits to estimate FAO numbers: 50% silking, the grain moisture content when that of the maturity group standards averaged 25% and 20%, and the grain moisture content at harvest.

In the present paper the accuracy of the two Hungarian methods of calculating maturity groups is compared with the German system and the GDD heat unit method.

## Materials and methods

The experiments were carried out in Martonvásár in the years 1999–2001. Each year tests were made on the official standards for each maturity group (FAO 200, 300, 400, 500), state registered Martonvásár hybrids, other major hybrids grown in Hungary, and experimental hybrids from Martonvásár (Table 1). The experiments were set up in two replications, with a plant density of 70,000 plants/hectare. The grain moisture content was determined in a drying cabinet.

The FAO numbers were determined as follows:

Old Hungarian method (Neduczáné Krékity, 1998): for each maturity group a linear regression line is plotted between the grain moisture at harvest of the two maturity group standards and their FAO numbers. The FAO number of each candidate variety can then be read off the graph based on their grain moisture contents.

New Hungarian method: using four parameters (50% silking and three grain moisture contents, measured at different times), as reported by Szieberth (2000) and Marton et al. (2001).

German method (according to Miltner and Rath, 1998):

$$\text{FAO number}_x = A + (B–C)/0.1$$

where FAO number$_x$ = the FAO number of variety x; A = average FAO number of the maturity group standards; B = average dry matter percentage of the maturity group standards; C = dry matter percentage of the variety

Heat unit calculation using the growing degree days (GDD) method (Gilmore and Rogers, 1958; Arnold, 1975):

$$\text{Heat unit} = (T_{max} - T_{min})/2 - 10$$

where $T_{max} = 30$, if $T_{max} > 30$; $T_{min} = 10$, if $T_{min} < 10$.

## Results and discussion

The grain moisture at harvest, which was the only parameter used to calculate FAO numbers in the earlier Hungarian and German systems, has a very narrow range of values (16.82–20.06%) (Table 1). Despite the low $LSD_{5\%}$ (1.1972) it is difficult to find hybrids within each maturity group that significantly differ from the mean of the standards in the given group. At the same time, there are considerable differences between the hybrids for flowering date (67.17–79.08 days).

*Table 1*
Flowering time, moisture content, FAO numbers and heat unit requirements of the hybrids (Martonvasar 1999–2001)

| Entry | Silking (day) | Moisture (%) | German FAO No. | Old Hung. FAO No. | New Hung. FAO No. | Heat units* | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | 30% | 25% | 20% |
| 01St1 FAO200 | 68.17 | 17.22 | 294 | 299 | 282 | 1190 | 1250 | 1332 |
| 02St2 FAO200 | 67.92 | 17.44 | 296 | 292 | 296 | 1203 | 1270 | 1375 |
| 03St3 FAO200 | 68.00 | 16.82 | 290 | 270 | 278 | 1201 | 1264 | 1348 |
| 04Exp1-200 | 67.17 | 16.90 | 291 | 274 | 271 | 1191 | 1252 | 1353 |
| 05Exp2-200 | 70.42 | 17.98 | 302 | 400 | 332 | 1239 | 1311 | nd. |
| 06Exp3-200 | 67.75 | 17.66 | 298 | 400 | 285 | 1211 | 1277 | 1366 |
| 07Exp4-200 | 68.17 | 17.50 | 298 | 356 | 298 | 1224 | 1299 | 1396 |
| 08Exp5-200 | 71.08 | 17.89 | 301 | 358 | 328 | 1247 | 1322 | 1439 |
| 09Exp6-200 | 70.83 | 17.95 | 301 | 402 | 313 | 1236 | 1302 | 1389 |
| 10Exp7-200 | 68.83 | 17.44 | 297 | 375 | 318 | 1243 | 1327 | 1439 |
| 11St3 FAO300 | 73.75 | 18.28 | 368 | 335 | 387 | 1292 | 1361 | 1460 |
| 12St1 FAO300 | 71.33 | 18.14 | 366 | 450 | 386 | 1283 | 1362 | 1476 |
| 13St2 FAO300 | 71.25 | 17.84 | 363 | 685 | 378 | 1291 | 1362 | 1460 |
| 14Exp1-300 | 73.25 | 17.70 | 362 | 641 | 401 | 1302 | 1373 | 1466 |
| 15Exp2-300 | 75.00 | 17.39 | 359 | 585 | 388 | 1297 | 1358 | 1434 |
| 16Exp3-300 | 71.67 | 17.88 | 364 | 711 | 427 | 1318 | 1397 | 1497 |
| 17Exp4-300 | 71.17 | 19.61 | 381 | 297 | 463 | 1339 | 1425 | 1541 |
| 18Exp5-300 | 70.67 | 18.90 | 374 | 386 | 448 | 1314 | 1402 | nd. |
| 19Exp6-300 | 71.58 | 19.38 | 383 | 732 | 492 | 1336 | 1414 | 1537 |
| 20Exp7-300 | 73.58 | 17.58 | 360 | 467 | 379 | 1299 | 1366 | 1460 |
| 21Exp8-300 | 71.42 | 17.51 | 360 | 776 | 399 | 1301 | 1381 | 1479 |
| 22St1FAO400 | 74.33 | 17.81 | 363 | 450 | 416 | 1316 | 1379 | 1456 |
| 23St2 FAO400 | 74.00 | 17.55 | 449 | 445 | 448 | 1316 | 1381 | 1469 |
| 24St1 FAO400 | 74.00 | 17.82 | 452 | 450 | 457 | 1336 | 1404 | 1485 |
| 25Exp1-400 | 74.00 | 18.86 | 462 | 559 | 509 | 1342 | 1421 | 1504 |
| 26Exp2-400 | 73.33 | 18.33 | 457 | 540 | 469 | 1342 | 1408 | 1490 |
| 27Exp3-400 | 72.33 | 19.44 | 468 | 578 | 511 | 1355 | 1428 | 1522 |
| 28Exp4-400 | 72.08 | 18.63 | 460 | 548 | 477 | 1324 | 1388 | 1465 |
| 29St3 FAO400 | 73.00 | 18.31 | 457 | 526 | 451 | 1319 | 1383 | 1468 |
| 30Exp5-400 | 72.92 | 18.63 | 460 | 511 | 507 | 1347 | 1415 | 1499 |
| 31Exp6-400 | 73.67 | 19.09 | 465 | 560 | 501 | 1338 | 1418 | 1519 |
| 32Exp7-400 | 73.83 | 18.18 | 456 | 507 | 481 | 1334 | 1406 | 1491 |
| 33Exp8-400 | 72.83 | 19.11 | 465 | 554 | 500 | 1350 | 1423 | 1517 |
| 34Exp9-400 | 73.58 | 19.00 | 468 | 611 | 501 | 1333 | 1436 | 1541 |
| 35Exp10-400 | 73.33 | 18.51 | 459 | 523 | 508 | 1361 | 1431 | 1515 |
| 36Exp11-400 | 73.92 | 17.61 | 450 | 459 | 458 | 1347 | 1422 | 1509 |
| 37Exp12-400 | 77.17 | 19.06 | 464 | 659 | 516 | 1363 | 1444 | 1538 |
| 38Exp13-400 | 73.42 | 20.82 | 482 | 720 | 554 | 1378 | 1471 | nd. |
| 39Exp14-400 | 77.92 | 19.39 | 468 | 562 | 512 | 1369 | 1433 | 1511 |
| 40Exp15-400 | 73.75 | 19 | 462 | 540 | 504 | 1370 | 1442 | 1530 |
| 41St1 FAO500 | 75.08 | 18.81 | 521 | 530 | 515 | 1362 | 1421 | 1500 |
| 42St2 FAO 500 | 74.67 | 19.20 | 522 | 635 | 531 | 1393 | 1454 | 1512 |
| 43Exp1-500 | 74.92 | 20.04 | 531 | 837 | 554 | 1375 | 1443 | 1522 |
| 44Exp2-500 | 76.67 | 19.32 | 523 | 630 | 474 | 1357 | 1413 | 1495 |
| 45Exp3-500 | 75.17 | 18.21 | 512 | 164 | 519 | 1369 | 1432 | 1503 |
| 46Exp4-500 | 79.08 | 19.06 | 521 | 298 | 516 | 1365 | 1414 | 1475 |
| 47St3 FAO 500 | 74.75 | 18.81 | 519 | 590 | 565 | 1389 | 1451 | 1522 |
| 48Exp5-500 | 78.00 | 19.70 | 527 | 846 | 613 | 1388 | 1455 | 1540 |
| 49Exp14-400 | 78.08 | 20.06 | 531 | 753 | 563 | 1440 | 1470 | 1553 |
| Mean | 72.91 | 18.43 | 416 | 512 | 442 | 1317 | 1388 | 1476 |
| $LSD_{5\%}$ | 1.324 | 1.197 | 12 | NS (359) | 57 | 37 | 41 | 53 |
| $LSD_{5\%}$ (%ofmean) | 2 | 6 | 3 | 70 | 13 | 3 | 3 | 4 |

NS: non-significant; nd: no data; *required to reach the given grain moisture content

Judging by the $LSD_{5\%}$ value (12) the German method of calculating FAO numbers appears to be extremely accurate. Hybrids that differ significantly from each other can be found in all the maturity groups with the exception of FAO 200. However, when two hybrids (St1 FAO400 and Exp14-400) were each tested in two different maturity groups, although there was no significant difference in the grain moisture contents recorded in the two groups (St1 FAO400: 17.81% and 17.82%; Exp14-400: 19.39% and 20.06%), this was not true of the FAO numbers (St1 FAO400: 363 and 452; Exp14-400: 468 and 531). This indicates that it was not the grain moisture values serving as the basis of the calculation that were unreliable, but the method of calculation that caused distortion. It thus appears that the German method may be misleading.

The $LSD_{5\%}$ value obtained with the new Hungarian method was substantially higher (57) and was only able to detect significant differences between the hybrids in FAO groups 400 and 500. The $LSD_{5\%}$ value obtained for the old Hungarian method (359) is greater than the whole of the maturity range grown in Hungary (260–550), so even the earliest and latest hybrids cannot be reliably distinguished. The F value of analysis of variance was not significant either.

Heat unit calculations were used to determine the heat units required to reach three different grain moisture contents: 20%, 25% and 30%. In all cases the lowest number of heat units was required by the FAO 200 hybrids to reach a given grain moisture content, and the highest number by the FAO 500 hybrids.

The difference between the FAO 200 and FAO 300 groups was greater than 80°C in all the grain moisture ranges. The difference between the FAO 300 and FAO 400 groups and between the FAO 400 and FAO 500 groups was 40°C for a grain moisture content of 30%, but declined at lower grain moisture contents. This confirms that in the case of late harvesting it is wise to choose the latest hybrids, provided the expected rainfall at the given location is sufficient to ensure that these hybrids fulfil their better yield potential. The correlation coefficients calculated between the FAO numbers obtained using various methods and the vegetation period parameters calculated in terms of heat units indicate that the new Hungarian FAO numbers can be converted into vegetation periods in terms of heat units with the greatest accuracy (Table 2). A knowledge of the new FAO number allows the heat unit requirements of the hybrids to be accurately predicted, while a knowledge of the heat unit requirements is a good indication of the FAO number.

*Table 2*
Correlation coefficients between the grain moisture at harvest, the FAO number and the heat units

| | Heat units required to reach the given grain moisture content | | |
|---|---|---|---|
| | 30% | 25% | 20% |
| Grain moisture | 0.782 | 0.806 | 0.804 |
| Old Hungarian FAO | 0.586 | 0.608 | 0.605 |
| German FAO | 0.925 | 0.888 | 0.796 |
| New Hungarian FAO | 0.968 | 0.963 | 0.911 |

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# EFFECT OF INCREASED UV-B RADIATION ON THE ANTHOCYANIN CONTENT OF MAIZE (*Zea mays* L.) LEAVES

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The level of UV-B radiation reaching the surface of the earth is increasing due to the thinning of the ozone layer in the stratosphere over recent decades. This has numerous negative effects on living organisms.

Some of the Hungarian inbred maize lines examined under the climatic conditions in Chile exhibited an unusually high proportion of pollen mortality, flowering asynchrony and barrenness. The evidence suggests that this can be attributed to the approx. 30% greater UV-B radiation in Chile.

The investigation of this problem within the framework of abiotic stress breeding programmes is extremely important in the light of the global rise in UV-B radiation, which may make it necessary to elaborate a selection programme to develop inbred lines with better tolerance of this type of radiation.

In the course of the experiment the same ten inbred lines, having different maturity dates and genetic backgrounds, were tested for five years in Chile and Hungary. The tests focussed on anthocyanin, a flavonoid derivative involved in the absorption of damaging UV-B radiation.

Averaged over years and varieties, the total anthocyanin content in the leaf samples was significantly higher in Chile than in Hungary. This was presumably a response at the metabolic level to the negative stress represented by higher UV-B radiation.

In the five early-maturing flint lines the anthocyanin contents were more than 45% greater than those recorded in Hungary. This suggests that these genotypes, originating from northern regions, were not sufficiently adapted to the higher radiation level. In these samples higher UV-B caused a sharp rise in the quantity of anthocyanin, which absorbs the dangerous radiation. In late-maturing genotypes the initial content of the protective compound anthocyanin was higher at both locations, so in these types the high radiation level was not a problem and did not cause any substantial change.

Similar conclusions were drawn from the results of fluorescence imaging. The F440/F690 ratio indicative of the stress level was higher in late lines with a high anthocyanin content, good tolerance and good adaptability.

**Key words:** *Zea mays* L., inbred line, ultraviolet-B radiation, flavone, anthocyanin, stress acclimation, fluorescence imaging

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## Introduction

One negative consequence of global climate change is the thinning of the ozone layer in the stratosphere, which functions as a shield against dangerous radiation. This has been caused in part by human activities, especially by the ozone-antagonistic gases emitted by heavy industry (Anderson et al., 1991; McFarland and Kaye, 1992; McKenzie et al., 1999; Reddy and Hodges, 2000).

A decrease of 1% in the ozone level may result in a 1.3–1.8% rise in the UV-B radiation (280–320 nm), the most biologically active form of ultraviolet radiation, with the most damaging effect on living organisms (Madronich, 1993). Naturally, the level of dangerous radiation depends on many factors, such as the latitude and altitude of the given location, the atmospheric conditions (cloud cover, fog, mist), the light reflection of the surface, etc. (Caldwell, 1981). Similar conclusions were drawn by Zerefos et al. (1997) when studying changes in the UV-B radiation in four European cities at various latitudes and altitudes over a number of years.

There can be little doubt that climatic changes are likely to cause a rise in the UV-B level in the future. In general, higher plants are able to tolerate a greater intensity of UV-B radiation than animals. Nevertheless, the development of plants capable of tolerating higher UV-B levels is soon likely to become an important part of plant breeding programmes aimed at overcoming abiotic stress factors (along with cold, drought, heat, salt, heavy metals).

Different plant species exhibit very varied sensitivity to UV-B radiation. In the course of evolution, natural selection led to the development of species capable of tolerating a higher UV-B level in locations where this was an ecological advantage. Many authors have investigated the effect of increased UV-B radiation on the morphological, ultrastructural, photochemical, biochemical and physiological parameters of various crops under field and greenhouse conditions, including peas (Strid et al., 1990), wheat (Teramura, 1980), soya (Teramura and Caldwell, 1981; Murali et al., 1988; Kramer et al., 1992), sugar beet (Bornman et al., 1983), turnip rape (Demchik and Day, 1996), beans (Cen and Bornman, 1990) and marrow (Sisson, 1981).

Very few papers are to be found, however, in which the experimental plant is maize, one of the world's most important crops. Investigations on the effect of increased UV-B radiation were reported by Hart et al. (1975) and Santos et al. (1998) on the duration of maize flowering, Pfahler (1981) on the germination ability of pollen and the growth of the pollen tube, Vu et al. (1982) and Santos et al. (1990) on the accumulation of carbohydrates, and Basiouny et al. (1978) and Santos et al. (1993) on the quantities of leaf chlorophyll and total soluble protein. Casati and Walbot (2003, 2005) and Long et al. (2003) emphasised the importance of the pigment and wax layers in the absorption of UV-B radiation by the maize epidermis. Barzig and Malz (2000) found that high UV-B radiation caused little damage to the leaves of sweetcorn, though the cell-

wall and membrane deformations observed in approx. 1/3 of the epidermal cell layers warned that there was a limit to this tolerance. Teramura and Sullivan (1994) listed maize as a species sensitive to UV-B radiation.

The responses of plants to more intense UV-B radiation occur mostly at the physiological and biochemical level (Hollósy, 2002), resulting in a reduction in radiation absorption on the leaf surface (Cen and Bornman, 1993). Morphological changes (such as leaf area reduction, leaf thickening, decrease in the number of stomata, etc.) may also take place in an attempt to avoid damage (Barnes et al., 1990; Tevini, 1993). In contrast, Santos et al. (1993) observed a thinning of the leaves as a consequence of greater exposure to UV-B. In another paper these authors mentioned that a higher level of UV-B radiation under artificial conditions delayed maize flowering by 2–3 days, significantly increased the quantity of radiation-absorbing pigments in the pollen wall and influenced the fertilising ability of the pollen (Santos et al., 1998). Walbot (1999) also reported that UV-B radiation had a negative effect on maize pollen, concluding, among other things, that the level of damage depended greatly on the duration of radiation. Experiments on transgenic Bt maize, however, demonstrated that a longer period of UV-B treatment had no effect on the toxicity of the pollen to insects (Ohlfest et al., 2002).

Intense UV-B radiation causes reactive oxygen radicals to be formed in the plants; these damage the membranes by inducing lipid peroxidation. In response, enzymes involved in the elimination of free radicals are activated (Arnott and Murphy, 1991; Dai et al., 1997), accompanied by a reduction in the leaf chlorophyll content, the quantity of Rubisco enzyme and the expression of genes involved in photosynthesis (Mackerness et al., 1997). The tolerance of higher plants to UV-B is substantially influenced by their antioxidant enzyme systems, which are capable of eliminating ROS before they can damage membranes (Mackerness, 2000).

Caldwell (1971) and other authors attributed the moderation of the damaging effect of UV-B radiation on epidermal and subepidermal cells to flavonoids and related compounds (such as anthocyanins). In response to increased UV-B radiation there is a rise in total phenols and total anthocyanins. Lois (1994) observed the synthesis of radiation-absorbing flavonoids (flavones, flavonols) after UV-B stress, and also that of proteins responsible for pathogenesis, which presumably play a role in the development of UV-B tolerance (Green and Fluhr, 1995). Plant genotypes such as *Arabidopsis* mutants, which are unable to accumulate flavonoids in the upper epidermal cells as a defence reaction, are extremely vulnerable to higher UV-B radiation (Landry et al., 1995). Polyamines and waxes also contribute to the development of UV tolerance (Kramer et al., 1992). The accumulation of anthocyanin in response to increased UV-B radiation was reported by Hashimoto et al. (1991), Singh et al. (1999) and other authors. In higher plants specific photoreceptors (e.g. cryptochromes) play an important role in the defence against UV-B radiation.

These regulate the biosynthesis of flavonoids and are thus indirectly involved in the formation of the anthocyanins responsible for absorption (Cashmore et al., 1999).

The present experiments were designed to detect quantitative changes in the formation of the anthocyanins involved in the defence mechanism, averaged over several maize genotypes grown at a higher level of UV-B radiation than that experienced in Hungary.

## Materials and methods

*Experimental material and sampling*

The same ten Martonvásár inbred maize lines (five early lines: L2-E, L3-E, L4-E, L9-E, L10-E, and five medium or late lines: L1-L, L5-L, L6-L, L7-L, L8-L) were sown in experiments with four replications in Martonvásár (Hungary) and Buin (Chile) between 2000 and 2004. Sampling was carried out during the last ten days of equivalent months (May, June and July in Martonvásár; November, December and January in Buin) after the plants had flowered. The 3–4th leaf under the tassel was removed from ten plants in each replication. Samples taken in Chile were packed in dry ice and sent to Hungary for processing within 24 hours, after which they were stored at –80°C until required.

*Spectrophotometric determination of anthocyanin content*

After removing the median leaf vein, 250 mg leaf material was rubbed in a mortar with quartz sand in 2 × 1 ml 1 N HCl-methanol, after which the plant extract was centrifuged at 4°C for 20 min at 12,000 *g*. The anthocyanin content was determined using a UV-VIS spectrophotometer (Shimadzu 160-A, Japan), calibrated with isolating solution. The absorbance of the supernatant containing the anthocyanins, recorded at 530 nm, was corrected with the non-specific absorbance recorded at 479 nm. The differences between these absorbance values ($A_{530}$–$A_{479}$/g fresh weight) are illustrated in the figures.

*Fluorescence imaging system*

A compact flash-lamp fluorescence imaging system was used to obtain fluorescence images of the leaves (Lichtenthaler and Babani, 2000). The fluorescence of intact leaves was excited by a Xenon flash lamp (FX-300UV) with the application of a broad UV transmitting filter (DUG 11, Schott, Mainz, Germany). The fluorescence bands F440 (i.e. near 440 nm), F520, F690 and F740 were selected by appropriate filters built into the camera (Photonetics GmbH, Kehl, Germany). Four hundred images were accumulated for each sample. The images were sensed on the adaxial (upper) side of the leaf, and were corrected by the background and by the inhomogeneity of the exciting light. They were then processed and stored by the image processing system Camille 1.05 (Photonetics GmbH, Kehl, Germany).

*UV-B radiation data and statistical evaluation*

UV-B radiation data were obtained from the Meteorological Services in Chile and Hungary. The AGROBASE'99 ANOVA program was used for the statistical evaluation (two- and three-factor analysis of variance).

## Results

The biologically active UV-B radiation recorded for the two experimental locations in Martonvásár (Hungary) and Buin (Chile) during three equivalent months of five experimental years differed significantly, being nearly 28%

higher on average in Chile. The greatest difference was observed in 2001, when the Chilean values were 37% higher than the corresponding Hungarian data. An analysis of the data revealed that the deviation was greater in Hungary, where considerable fluctuation was observed between the daily data. This was not the case in Chile, presumably due to the complete lack of cloud cover during the relevant months (Fig. 1).

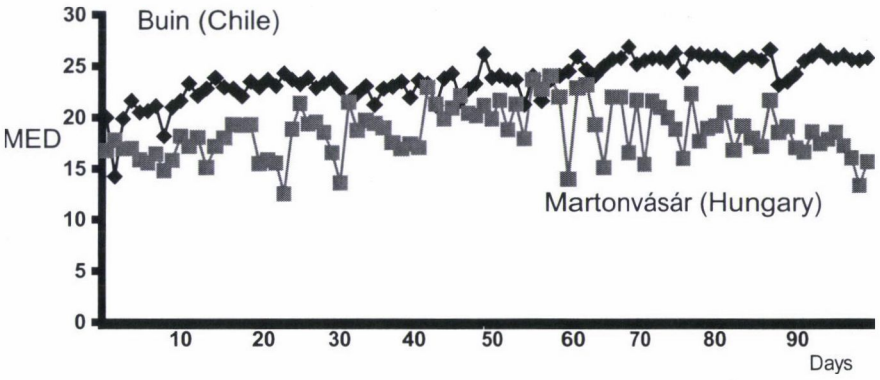



*Fig. 1.* Mean course of the daily sums of biologically effective UV irradiation on the basis of 5 years (2000–2004) in Buin (Chile) in November, December and January and in Martonvásár (Hungary) in May, June and July. MED: minimum erythema dose

In all five years the same ten inbred maize lines were tested (five early lines: L2-E, L3-E, L4-E, L9-E, L10-E, and five medium or late lines: L1-L, L5-L, L6-L, L7-L, L8-L). The following conclusions could be drawn from the results of two- and three-factor analysis of variance. The total anthocyanin content in the Chilean samples was significantly (16%) greater, averaged over ten lines and five years, than in the Hungarian samples (Buin: $A_{530-479} = 0.555$, Martonvásár: $A_{530-479} = 0.478$) (Table 1).

The MS values for three-factor ANOVA indicate that the location had the greatest effect on the anthocyanin content of the leaves, followed by the variety, while the year had the smallest influence on the traits tested. Among the interactions, the most significant was the variety × location, followed by the variety × year and the year × location. For the traits examined, the main effects of the factors and the interactions were significant at the P = 0.1% level. Figure 2 illustrates the total anthocyanin contents recorded in the various years at the two locations. In all five years the mean values of the Chilean samples were higher, suggesting that the negative effect of the significantly higher biologically active UV-B radiation led to responses at the metabolic level in maize. Anthocyanin synthesis was activated by higher levels of UV-B radiation to some extent in all the genotypes. In late inbred lines, where the basic value of total anthocyanin

content is high, only a slight, non-significant increase was observed, while the five early genotypes (L2-E, L3-E, L4-E, L9-E, L10-E), which belonged to the F 2, CM 7, etc. gene pools, exhibited a very significant rise in the total anthocyanin content in response to higher levels of UV-B radiation, averaged over five years. In these genotypes the Chilean values were significantly higher than the Hungarian values every year and it was here that flowering asynchrony and barrenness were frequently observed in Chile. In some years it proved impossible to carry out variety maintenance on certain lines due to the lack of silks.

A detailed analysis of the data in Table 1 indicated that the late-maturing lines had very similar, high values of total anthocyanin at both locations, averaged over five years, the highest value being recorded for the latest line, L1-L.

Among the early-maturing lines, some exhibited similar responses every year (e.g. L2-E, L4-E), while for others, such as L9-E and L10-E, the deviation between the annual values was considerably greater. This suggests that there are considerable differences between the genotypes for their response to UV-B radiation. Averaged over years and locations (Fig. 3) the mean increase observed spectrophotometrically for these early genotypes was over 45% higher in Chile than in Hungary. For line L2-E the increase in total anthocyanin content in Chile was more than 71%, and even for lines L4-E, L9-E and L10-E it was in excess of 40%.

*Table 1*

Differences in absorbances ($A_{530}$–$A_{479}$/g fresh weight) characteristic of the anthocyanin content of maize inbred lines at each location averaged over the experimental years

| No. | Lines | Martonvásár (Hungary) | | | | | | Buin (Chile) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Years | | | | | | Years | | | | | |
| | | 2000 | 2001 | 2002 | 2003 | 2004 | Mean* | 2000 | 2001 | 2002 | 2003 | 2004 | Mean* |
| 1 | L1-L | 0.566 | 0.800 | 0.680 | 0.659 | 0.684 | 0.678 | 0.776 | 0.616 | 0.675 | 0.679 | 0.622 | 0.674 |
| 2 | L2-E | 0.257 | 0.229 | 0.238 | 0.312 | 0.293 | 0.266 | 0.358 | 0.463 | 0.350 | 0.578 | 0.536 | 0.457 |
| 3 | L3-E | 0.371 | 0.357 | 0.434 | 0.301 | 0.681 | 0.429 | 0.381 | 0.523 | 0.512 | 0.660 | 0.608 | 0.537 |
| 4 | L4-E | 0.308 | 0.387 | 0.209 | 0.430 | 0.311 | 0.329 | 0.568 | 0.580 | 0.542 | 0.420 | 0.291 | 0.480 |
| 5 | L5-L | 0.692 | 0.863 | 0.731 | 0.672 | 0.730 | 0.737 | 0.564 | 0.809 | 0.474 | 0.640 | 0.587 | 0.615 |
| 6 | L6-L | 0.223 | 0.674 | 0.501 | 0.460 | 0.704 | 0.513 | 0.428 | 0.734 | 0.370 | 0.566 | 0.429 | 0.506 |
| 7 | L7-L | 0.390 | 0.590 | 0.685 | 0.479 | 0.333 | 0.495 | 0.621 | 0.447 | 0.528 | 0.569 | 0.464 | 0.526 |
| 8 | L8-L | 0.472 | 0.860 | 0.604 | 0.344 | 0.474 | 0.551 | 0.595 | 0.754 | 0.520 | 0.682 | 0.657 | 0.641 |
| 9 | L9-E | 0.473 | 0.206 | 0.390 | 0.454 | 0.423 | 0.407 | 0.660 | 0.562 | 0.483 | 0.566 | 0.639 | 0.582 |
| 10 | L10-E | 0.333 | 0.436 | 0.385 | 0.338 | 0.366 | 0.372 | 0.440 | 0.681 | 0.639 | 0.476 | 0.384 | 0.524 |
| | Mean+ | 0.409 | 0.540 | 0.486 | 0.454 | 0.500 | 0.478 | 0.539 | 0.617 | 0.509 | 0.583 | 0.522 | 0.554 |

*: mean of lines; +Mean of years; $LSD_{5\%}$=0.023 (between lines), $LSD_{5\%}$=0.010 (between locations), $LSD_{5\%}$=0.016 (between years)

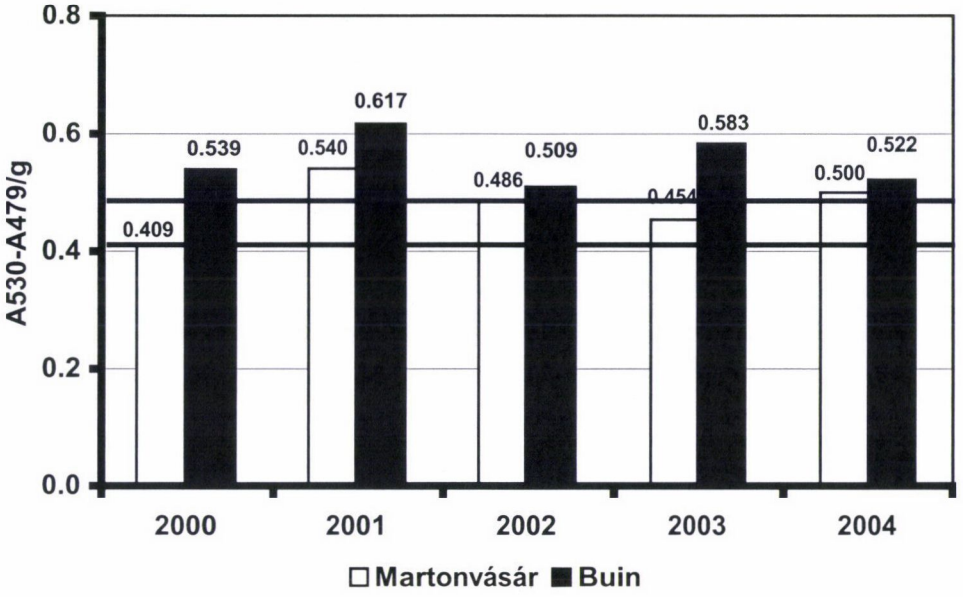



*Fig. 2.* Absorbance values of ten lines averaged over five experimental years at each location (Martonvásár, Buin)

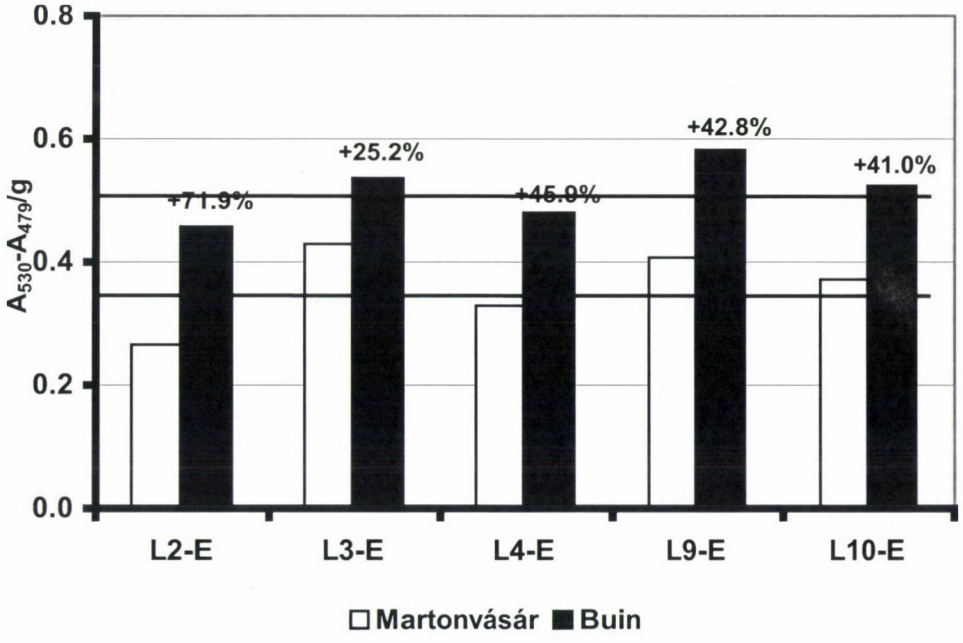



*Fig. 3.* Absorbance values ($A_{530}-A_{479}$/g) of five early-maturing inbred maize lines, averaged over three experimental years (2000–2002) at each location

The results indicate that some genotypes have a higher basic anthocyanin content (such as the late genotypes in the present experiments), giving them an automatic defence against higher UV-B radiation, which explains the lack of a clear response. In the present experiments it was these lines that proved to be more stress-tolerant. This was confirmed by the results of fluorescence imaging at several wavelengths, indicative of the physiological status of the plants. The high $F_{440}/F_{690}$ ratios obtained for the five late-maturing lines (not shown) showed that they contained a larger quantity of compounds fluorescing in the blue range of the spectrum (probably including the anthocyanins important for the defence system). These changes can be interpreted as stress acclimation. Other genotypes, mainly early-maturing lines, have a much lower anthocyanin content in the leaves, which rises abruptly in response to a higher level of UV-B radiation. However, this does not necessarily make the lines more tolerant of stress, as illustrated by our experiences in Chile. The $F_{440}/F_{690}$ ratio, indicative of the level of stress, was considerably lower in these genotypes, indicating their poorer ability to acclimatise.

## Discussion

The results of five years of experimentation demonstrate that the content of anthocyanin, a compound playing an important role in radiation absorption, in the leaves of inbred maize lines was higher in Chile, where there is a higher level of UV-B radiation, than in Hungary, where this level is significantly lower. This is in agreement with the conclusions of Chappell and Hahlbrock (1984) and the rice scientists Tevini et al. (1991), who demonstrated the formation of flavonoid pigments capable of absorbing higher UV-B radiation in the epidermal cells. The same conclusion was drawn by Santos et al. (1993), who reported increases in five soluble proteins during the electrophoresis of hybrid maize leaf samples exposed to higher levels of UV-B radiation. This suggested that in maize plants UV-B radiation stimulated the synthesis of enzymes involved in the formation of flavonoids. Electron microscopic analysis revealed no damage to the leaf cells, which can presumably be attributed to the enhanced flavonoid level. Conclusions similar to those drawn in the present work are also suggested by the data presented by Santos et al. (1998), who found that in response to intense radiation pollen grains exhibited a significant increase in the quantity of pigments capable of absorbing UV-B radiation. The absorbance was 30% greater in treated plants. Among the ten inbred lines examined in the present work, the absorbance values recorded for the five early lines were 43% higher in the Chilean samples, averaged over 5 years.

Inbred maize lines with different genetic backgrounds and different maturity dates were examined in the present experiments. The very diverse absorbance data obtained for these varieties confirm the results of Semeniuk and Stewart (1979) and Murali et al. (1988), who found differences in UV-B

sensitivity not only between plant species, but also between varieties. Data obtained over a number of years demonstrate that there are substantial differences between maize genotypes in the synthesis of UV-B-absorbing anthocyanins and in the responses given to UV-B stress. The data could be of assistance in the selection of genotypes capable of tolerating higher levels of UV-B radiation for use in stress resistance breeding.

The present studies did not cover the morphological, ultrastructural and other physiological changes caused by increased UV-B radiation, being limited to the analysis of anthocyanin synthesis, a flavonoid involved in plant defence mechanisms as an antioxidant and radiation absorber. The fluorescence imaging method applied in the present work was found to be ideally suited for detecting the exposure of plants to stress. This technique provides valuable additional information when used in combination with other methods for determining the level of photosynthesis (e.g. infrared gas analysis, chlorophyll-a fluorescence induction measurements, determination of total chlorophyll content, etc.). It is planned to continue these studies under field conditions in Chile and Hungary, using more genotypes with more diverse genetic backgrounds, and also under artificial conditions in a phytotron.

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# PARTICIPATORY SORGHUM VARIETAL EVALUATION AND SELECTION IN PAKISTAN

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A number of improved pipeline varieties of sorghum including PARC SS-1, PARC SS-2 and PARC SS-15 were planted in various agro-ecological zones of Pakistan for 2 years (2004 and 2005). The participatory Mother-Baby Trial methodology was used for the first time in Pakistan for varietal evaluation and selection. Six varieties of sorghum were evaluated along with the local checks. In general, the pipeline varieties developed at the National Agricultural Research Centre had a yield advantage of 15 to 20% over the local checks and other improved varieties included in the trial. The farmers were involved in the varietal selection process. These varieties were selected by the farmers on the basis of maturity, higher yield, resistance to leaf blight and red rot diseases, drought tolerance, and the dual (grain cum fodder) nature of the varieties. Based on the evaluation and selection, these varieties are being released for general cultivation in various ecologies. The results from participatory trials on various agronomic aspects are presented and discussed in this paper.

**Key words**: sorghum, participatory, evaluation, selection, Pakistan

## Introduction

In Pakistan, sorghum is an important coarse grain crop in dryland ecologies, including the rainfed areas of Pakistan. It is an important source of food, feed and fodder (Gomes et al., 1992). Sorghum is cultivated for both food grain and as a forage crop, and about 90% of production is consumed on-farm as food and seed (Chughtai et al., 2004). The sorghum area is equally divided between rainfed and irrigated cultivation, and at least one quarter of the rainfed and half of the irrigated crop is grown mainly for forage use.

Sorghum production in Pakistan has literally remained unchanged for the last three decades (Chughtai et al., 2004). During the same period, the sorghum area has declined while the yield has marginally increased. The ratio of sorghum production used for human food consumption has also remained stagnant (around

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85%) and now this use is declining. Currently about 80% of total sorghum production is used for food and about 7% goes into animal feed. It is expected that while its direct use as human food may decrease, its indirect use for livestock feed will increase considerably (Hash, 1994). Also, its industrial use is likely to increase. It will be used much more extensively as green fodder and also as dry fodder, since no other crop can replace it for this purpose (Hash, 1994). Currently, in Pakistan sorghum is the most important green fodder during the summer season. Sorghum has a nutritional value comparable to maize, the other coarse grain crop. Sorghum is an important cereal for the hottest and driest areas where rainfed agriculture is practised. It is one of the most reliable producers of biomass and grain in these difficult, delicate and highly unpredictable environments due to its rapid growth rate, short duration of growth, tremendous developmental plasticity and adaptation to high temperatures and low soil fertility (Rachier and Majumdar, 1980).

Sorghum is the fourth most important cereal in Pakistan after wheat, rice and maize. It is annually grown on about 0.31 million hectares with a total production of 0.19 million tonnes and a yield of 606 kg $ha^{-1}$ (FAO, 2005). This yield is substantially lower compared to other regions. Even in Pakistan, yields up to four or five times higher have been obtained in experimental plots and on the fields of progressive farmers. This yield gap can be attributed to a number of factors. However, the lack of improved production technology, high-yielding improved varieties and quality seed have been identified as the most critical factors limiting the production of sorghum in rainfed areas.

During the last two decades, research efforts in various public research institutes have resulted in the development and identification of high-yielding, drought-tolerant cultivars of sorghum. These have the potential to replace the local low-yielding varieties cultivated by farmers (Naeem et al., 1990; 1992; Akmal et al., 1992; Shakoor, 1994; 1995). Also, the crop is cultivated by farmers using conventional methods.

There is a need for on-farm verification of appropriate germplasms and developed varieties of sorghum adapted to the dry ecology of rainfed areas. In addition, the introduction and adoption of an improved technology and its popularization among farmers through on-farm demonstrations and verifications may serve as a catalyst to increase productivity, as has been proved in many other cases (Tabo et al., 1999a, b).

The rate of adoption of the improved production technology and improved varieties of sorghum is very slow, and practically non-existent in dryland areas. It is obvious that improved seeds developed by researchers are not readily available to the farmers. Farmers keep their own seed year after year. The yield potential of the farmers' varieties deteriorates over time. Also, the farmers plant their crops under unimproved traditional cultural practices. Therefore, to address the problem of improved technology development and quality seed, a project activity was undertaken to initiate participatory research in selected districts of Pakistan. The selected districts represent the areas where sorghum is the most important cereal crop.

The participatory research approach adopted in the project involved the collaboration of farmers and scientists in agricultural research and development (Bentley, 1994). The project involved the participation of farmers, researchers and extension workers collaborating in a group. Participatory on-farm varietal evaluation and selection activities in several countries are yielding excellent results because the interventions are readily adopted by the farmers (Bellon, 2001; Gautman et al., 2002; Tiwari et al., 2002).

Under this project, the participatory Mother-Baby trial methodology was used for the first time in sorghum variety evaluation and selection. Three pipeline varieties developed by the National Agricultural Research Centre (NARC), three released improved varieties developed by other regional research centres along with the local checks were evaluated at six different locations across the country. Based on the results of the project, varieties with outstanding performance, developed by NARC and selected by farmers, are now being approved and released for general cultivation by the sorghum growers in Pakistan.

## Materials and methods

For evaluation, three improved varieties, namely YSS-9, DS-97 and RARI S-3, released by the Maize and Millet Research Institute, Yousafwala, the Agricultural Research Institute, D.I. Khan and the Regional Agriculture Research Institute, Bahawalpur, respectively, were included in the trials. Three promising pipe-line varieties (PARC SS-1, PARC SS-2 and PARC SS-15), developed by the National Agricultural Research Centre, Islamabad, were also included in the trials. The participatory trials were planted at six locations: Islamabad, Chakeal, D.I. Khan, Bahawalpur, Umerkot and Dadu. The mother-baby trial methodology was adopted for the first time in Pakistan for the evaluation and selection of sorghum varieties planted in different ecologies. At each location, the improved variety already planted by the farmers of the area was included as a check for comparison. The mother trials were replicated thrice and planted in a randomized complete block design at the research stations. They were managed by the researchers and were visited by the farmers at different stages. The data were collected and selections were made by the technical staff and the scientists. The farmers were also asked to make their own selections. The baby trials were two non-replicated subsets of the mother trials, generally planted in farmers' fields and managed by the farmers themselves under the supervision of a scientist. The farmers were asked to select the variety(ies) which fulfilled their requirements. At each location the varieties were grown in plots with four 5-m rows, with 75 cm row to row and 15 cm plant to plant distances. Fertilizers were applied at a rate of 90-45-0 kg NPK $ha^{-1}$. Half of the N with the full dose of $P_2O_5$ was incorporated in the soil at planting and the other half was applied when the crop was at the six-leaf stage. For plant protection, Furadon Granules was used @ 16 kg $ha^{-1}$ to control stem borer and shoot fly. Primextra @ 0.75 litre a.i. $ha^{-1}$ was used for weed control. Grain yield was recorded from the middle two rows of each plot, measuring 7.5 $m^2$. Grain yields were calculated at 15% moisture level. Data were recorded on days to 50% flowering, plant height, grain and stover yields, disease and insect resistance. The data were statistically analysed using analysis of variance and the means were compared by Duncan's Multiple Range Test (Steel and Torrie, 1960).

## Results and discussion

The data on three agronomic characteristics, i.e. grain yield, days to 50% flowering and plant height, from six different locations are presented in Tables 1 to 3. The data on stover yield and disease and insect reactions were not recorded at all the locations, so they are not presented in this manuscript. Data from the non-replicated baby trials are also omitted.

In 2004, the promising variety PARC SS-1, developed by NARC, gave a 10% higher mean grain yield, while PARC SS-1 was at par for yield with the local checks (which were improved varieties grown by the farmers). The released varieties developed by other research institutes were in fact outyielded by the local checks (Table 1) for mean grain yield, although the results are evidently location-specific. The results from the mother trials in 2005 (Table 1) indicate that the yields of the NARC pipeline varieties and the released varieties were superior to those of local checks. The NARC varieties outyielded the local checks by 12 to 33% for grain yield (Table 1).

*Table 1*

Mean grain yield (kg $ha^{-1}$) in improved varieties of sorghum at different locations in Pakistan during 2004 and 2005

| Varieties | NARC, Islamabad | AZRI, Umarkot | ARI, D.I. Khan | RARI, Bahawalpur | BARI Chakwal | ARI, Dadu | Mean | % of check |
|---|---|---|---|---|---|---|---|---|
| 2004 | | | | | | | | |
| PARC SS-1 | 2133 | 2439 | 3083 | 3444 | 1754 | 3389 | 2707 | 110 |
| PARC SS-2 | 2267 | 1761 | 3028 | 2750 | 1600 | 3333 | 2457 | 100 |
| PARC SS-15 | 2687 | 1409 | 1722 | 2833 | 1811 | 2722 | 2197 | 89 |
| YSS-9 | 1867 | 1075 | 1761 | 2667 | 2141 | 3083 | 2099 | 85 |
| RARI S-3 | 1289 | 2154 | 3000 | 3028 | 1961 | 2195 | 2271 | 92 |
| DS-97 | 1600 | 1165 | 2833 | 2833 | 1875 | 2917 | 2204 | 90 |
| Local check | 934 | 1409 | 3333 | 2500 | 1336 | 3139 | 2462 | 100 |
| CV (%) | 18.11 | 16.5 | 18.82 | 8.31 | 13.6 | 16.17 | | |
| LSD (5%) | 588 | 800 | 897 | 424 | 733 | 853.6 | | |
| 2005 | | | | | | | | |
| PARC SS-1 | 2933 | 2693 | 2611 | 2800 | 2754 | 2267 | 2676 | 133 |
| PARC SS-2 | 2622 | 2000 | 3444 | 2533 | 2333 | 2556 | 2581 | 128 |
| PARC SS-15 | 2356 | 1800 | 2778 | 2133 | 2133 | 2250 | 2242 | 112 |
| YSS-9 | 2400 | 1367 | 1278 | 2000 | 1867 | 2278 | 1865 | 93 |
| RARI S-3 | 2311 | 2233 | 1278 | 2178 | 2000 | 2195 | 2033 | 101 |
| DS-97 | 1756 | 1567 | 3055 | 2000 | 2267 | 2355 | 2117 | 105 |
| Local check | 1333 | 1433 | 3222 | 2000 | 1567 | 2500 | 2009 | 100 |
| CV (%) | 26.78 | 15 | 17 | 9 | 13.5 | 9 | | |
| LSD (5%) | 782 | 815 | 766 | 361 | 700 | NS | | |

NS = non-significant

The improved varieties already released (YSS-9, RARI S-3 and DS-97) and cultivated by farmers in different areas generally had better yield performance in their specific areas of adaptability (Table 1). For example, DS-97, developed at the Agricultural Research Station D.I. Khan, was particularly high-yielding in this area. Similarly, RARI S-3, developed at the Regional Agricultural Research Institute (RARI) Bahawalpur, was well adapted to the Bahawalpur area. YSS-9, specifically developed for irrigated areas, was significantly outyielded by the local checks and by the NARC pipeline varieties, since most of the trials were planted in ecologies with high temperature and low rainfall. In contrast, the NARC pipeline varieties (PARC SS-1, PARC SS-2 and PARC SS-15) performed well in all the major sorghum-growing areas of the country. PARC SS-1 gave the highest mean grain yield in both the years and was closely followed by PARC SS-2 (Table 1). The stalk yield data at harvest (not presented) also indicated the superior performance of the pipeline varies over the local checks and released varieties. RARI S-3, PARC SS-1 and PARC SS-15 had 25%, 24% and 23% higher stover yield than the checks, respectively. The stalk yield data, although not available from all the test locations, indicate that the pipeline varieties are dual purpose, that is, they can give good grain and stover yields.

The data on the days to 50% flowering (Table 2) indicated that in general the NARC pipeline varieties and other improved varieties matured later than the local checks. In particular, the variety developed for irrigated areas (YSS-9) was very late maturing and will not be suitable for rainfed ecologies. The plant heights of the NARC varieties and other improved varieties were relatively lower than the local checks (Table 3). YSS-9 is, however, the tallest variety and may thus be more susceptible to lodging and stem breaking.

Based on these results, it is concluded that the pipeline varieties developed at the National Agricultural Research Centre are not only high yielding, but are also widely adapted to the sorghum-growing areas of the whole country. Until very recently, the sorghum variety development in Pakistan was done mainly for high yield and other related characters. The preferences of farmers were not generally considered during the selection process. Consequently, the varieties released have not been adopted by farmers on a large scale. The participatory approach, involving evaluation and selection, was adopted for the first time in the current studies in order to consider the characteristics preferred by farmers. The field days were conducted so that large number of farmers could visit, evaluate and select the desired genotypes from the mother and baby trials. The researchers also evaluated the genotypes and selected the desired ones on the basis of grain and stover yields, maturity, dual grain and fodder production, stay-green characteristic, drought tolerance, insect and disease resistance, and other associated characters. The farmers believed that the pipeline varieties developed by NARC were drought tolerant. Another outstanding characteristic pointed out by farmers was the ease with which the improved varieties could be threshed by

hand. The large grain size and medium height of the improved pipeline cultivars were acceptable to the farmers. The fodder of the improved pipeline cultivars was considered more succulent and palatable than that of the local variety. PARC SS-1, as a stay-green variety, has the advantage of higher stalk yield, better fodder quality and extended duration. However, because of the semi-compactness of the panicles, the pipeline varieties were more susceptible to insect damage and bird attack. Participating and non-participating farmers were excited about the performance of improved pipeline varieties of sorghum. They exchanged seeds of these varieties among themselves to sow in the coming season.

Under the same project, the varietal evaluation and selection of pearl millet has also yielded excellent results and one of the NARC pipeline varieties has been approved for general cultivation in the rainfed areas of Pakistan. The findings of the project are in general agreement with several similar studies and have convincingly proved the effectiveness of the participatory approach for sorghum improvement and adoption (Tabo et al., 1999a, b).

*Table 2*

Mean days to 50% flowering in improved varieties of sorghum at different locations in Pakistan during 2004 and 2005

| Varieties | NARC, Islamabad | AZRI, Umarkot | ARI, D.I. Khan | RARI, Bahawalpur | BARI Chakwal | ARI, Dadu | Mean | % of check |
|---|---|---|---|---|---|---|---|---|
| 2004 | | | | | | | | |
| PARC SS-1 | 74 | 57 | 64 | 98 | 63 | 105 | 77 | 115 |
| PARC SS-2 | 69 | 55 | 62 | 96 | 59 | 79 | 70 | 104 |
| PARC SS-15 | 75 | 56 | 68 | 104 | 58 | 89 | 75 | 112 |
| YSS-9 | 73 | 57 | 72 | 100 | 60 | 111 | 79 | 118 |
| RARI S-3 | 73 | 55 | 75 | 91 | 54 | 104 | 75 | 112 |
| DS-97 | 73 | 56 | 53 | 80 | 60 | 112 | 72 | 107 |
| Local check | 70 | 57 | 52 | 69 | 55 | 99 | 67 | 100 |
| CV (%) | 8.35 | 8.4 | 1.48 | 16.5 | 12.8 | 7.94 | | |
| LSD (5%) | 10.77 | 8.5 | 1.68 | 8.3 | 6.5 | 14.10 | | |
| 2005 | | | | | | | | |
| PARC SS-1 | 68 | 53 | 68 | 89 | 64 | 84 | 71 | 106 |
| PARC SS-2 | 64 | 57 | 65 | 92 | 61 | 82 | 70 | 104 |
| PARC SS-15 | 71 | 53 | 71 | 73 | 59 | 86 | 69 | 103 |
| YSS-9 | 75 | 56 | 67 | 89 | 62 | 86 | 73 | 109 |
| RARI S-3 | 72 | 55 | 74 | 78 | 56 | 86 | 70 | 104 |
| DS-97 | 56 | 55 | 63 | 85 | 59 | 87 | 68 | 101 |
| Local check | 59 | 56 | 62 | 89 | 54 | 84 | 67 | 100 |
| CV (%) | 2.52 | 6.8 | 8.11 | 10.2 | 8.6 | 3.00 | | |
| LSD (5%) | 2.97 | NS | NS | 13.6 | 6.1 | NS | | |

NS = non-significant

*Table 3*
Mean plant height (cm) in improved varieties of sorghum at different locations in Pakistan during 2004 and 2005

| Varieties | NARC, Islamabad | AZRI, Umarkot | ARI, D.I. Khan | RARI, Bahawalpur | BARI Chakwal | ARI, Dadu | Mean | % of check |
|---|---|---|---|---|---|---|---|---|
| 2004 | | | | | | | | |
| PARC SS-1 | 115 | 195 | 155 | 174 | 189 | 128 | 159 | 93 |
| PARC SS-2 | 113 | 215 | 150 | 140 | 173 | 113 | 151 | 88 |
| PARC SS-15 | 157 | 197 | 238 | 165 | 111 | 147 | 169 | 99 |
| YSS-9 | 153 | 215 | 257 | 153 | 161 | 128 | 178 | 104 |
| RARI S-3 | 147 | 219 | 210 | 156 | 104 | 142 | 163 | 95 |
| DS-97 | 135 | 227 | 163 | 150 | 120 | 142 | 156 | 91 |
| Local check | 147 | 219 | 175 | 162 | 206 | 116 | 171 | 100 |
| CV (%) | 20.91 | 18.3 | 4.53 | 16.5 | 13.2 | 19.57 | | |
| LSD (5%) | 51.20 | 30.0 | 15.51 | 20.0 | 18.5 | 80.84 | | |
| 2005 | | | | | | | | |
| PARC SS-1 | 140 | 102 | 160 | 120 | 183 | 155 | 143 | 79 |
| PARC SS-2 | 130 | 166 | 142 | 123 | 165 | 181 | 151 | 83 |
| PARC SS-15 | 190 | 120 | 220 | 174 | 126 | 202 | 172 | 95 |
| YSS-9 | 203 | 158 | 230 | 156 | 168 | 201 | 186 | 102 |
| RARI S-3 | 182 | 176 | 210 | 154 | 116 | 216 | 176 | 97 |
| DS-97 | 152 | 181 | 188 | 150 | 110 | 166 | 158 | 87 |
| Local check | 198 | 148 | 208 | 142 | 205 | 189 | 182 | 100 |
| CV (%) | 6 | 13 | 5 | 12 | 18 | 20 | | |
| LSD (5%) | NS | 24 | 16 | 24 | 32 | NS | | |

NS = non-significant

The participatory on-farm evaluation of sorghum cultivars, a part of a larger project study, facilitated the selection of pipeline varieties by farmers who were enthusiastic to grow them before their release. Based on these and some earlier evaluations, the sorghum cultivar PARC SS-1 has been very recently approved by the Variation Evaluation Committee of the Pakistan Agricultural Research Council, Islamabad under the name of "Johar". The varieties have been recommended and released for large scale production and adoption by farmers in the low-rainfall areas of the country. The enthusiasm of the farmers has certainly enhanced the release process of these varieties. Under the project, large-scale seed production and distribution is under way and this is likely to improve the productivity of sorghum, especially in the rainfed areas of Pakistan.

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# MAIZE POPULATION HYBRIDS: SUCCESSFUL GENETIC RESOURCES FOR BREEDING PROGRAMS AND POTENTIAL ALTERNATIVES TO SINGLE-CROSS HYBRIDS

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Conservation of maize (*Zea mays* L.) genetic resources has been the emphasis of national and international institutions for the benefit of mankind. However, limited resources have been devoted to their adequate exploitation, making genetic resources less useful to the public and private scientific community. As a consequence, public maize breeders have exploited a limited number of heterotic combinations for cultivar development and basic molecular studies while genetic effects are different for different hybrids. Extensive testing of maize population hybrids is a successful approach to choose and improve germplasm sources with high mean performance, useful genetic variability, and excellent combining ability. There is a need to keep applied breeding programs strong in order to link efforts in germplasm conservation with its improvement and utilization.

**Key words:** germplasm choice, germplasm utilization, heterosis, maize, population hybrids

## Introduction

### *Justification for an adequate exploitation of genetic resources*

The balance between conservation of plant genetic resources and their effective use was addressed for the first time by the International Treaty on Plant Genetic Resources for Food and Agriculture in June 2006. Fortunately, exploitation of maize germplasm is still possible due to long-term improvement efforts on diverse and useful genetic resources that have been conducted by public breeding programs. The current reduction in the number of applied breeding programs and their capacity and resources (Frey, 1996; Sonino et al., 2006), however, limit the ability of the breeder to screen and improve genetic resources adequately.

Public U.S. maize breeding programs are under serious erosion because of lack of funding. This loss of mostly public support affects breeding continuity,

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objectivity and, equally important, the training of the next generation of maize breeders and the utilization and improvement of maize germplasm. Genetically broad-based maize public germplasm has significantly been utilized and recycled by industry. B73 was developed from an improved version of the genetically broad-based synthetic variety Iowa Stiff Stalk Synthetic (BSSS) and it has produced billions of dollars without intellectual property rights.

It is essential to perform extensive testing in order to choose and improve germplasm sources with high mean performance, useful genetic variability, and excellent combining ability. Most of the efforts on breeding programs with population improvement have included improvement of populations *per se* but limited testing of elite population hybrids. Maize population hybrids are crosses between divergent populations that have undergone long-term selection and are competitive with single-cross commercial hybrids (Carena and Wicks, 2006). Therefore, the 'population-hybrid concept' (Carena, 2005b) is an alternative that utilizes genetically broad-based germplasm to exploit heterosis. To date, the identification of alternative heterotic patterns has not been successful due to poor choices of germplasm, the lack of extensive testing and germplasm improvement, and the concentration of breeding efforts on specific genetic backgrounds. The number of successful heterotic combinations currently available is limited because current and useful public genetic diversity in reserve (Duvick, 1984) has not been fully exploited (Hallauer and Miranda, 1988) and identified. In addition, few reciprocal recurrent selection programs have been developed and utilized for this purpose, especially under extensive testing (Hallauer and Eberhart, 1970; Hallauer et al., 1988; Coors, 1999; Carena and Hallauer, 2001).

*Breeding continuity: The North Dakota State University (NDSU) example*

*History*

Maize was the first agricultural crop grown in ND (Olson et al., 1927) in environments characterized by very short growing seasons, low annual rainfall, and limited growing degree units. More than 300 years ago the women of agricultural tribes of Native Americans (Mandan, Arikara and Hidatsa) living in the Upper Missouri Valley were the first 'maize breeders' (Olson and Walster, 1932). These plant breeders and the 'corn mother' (defined as the woman that never dies) produced diverse strains of maize varieties. Native Americans selected genetic material adapted to the challenging ND environmental conditions. These open-pollinated (OP) varieties derived from NDSU and other land-grant Universities constituted the source of maize germplasm at the time the ND Agricultural College was founded in 1890. State maize variety tests began in 1892. The most popular varieties were Falconer, Northwestern Dent and Minnesota 13 (Olson and Walster, 1932). Mass selection and ear-to-row breeding methods as well as self-fertilization were popular improvement efforts

with emphasis on selection for early maturity and cold tolerance. Mass selection, defined at that time as 'simple selection' (Olson et al., 1927), was performed by selecting ears after harvest (Method I) and selecting ears from the best plants in the field (Method II). The latter tried to answer the same questions addressed by Gardner (1961) when trying to reduce environmental bias by selecting plants with stiff stalks and healthy leaves before looking at the ear. The ear-to-row selection method resulted in rapid improvement of non-adapted varieties (Olson et al., 1927) but no yield improvements were obtained by continuous ear-to-row selection until the modification of the method was used (Lonnquist, 1964; Hyrkas and Carena, 2005). Self-fertilization resulted in different strains and allowed breeders to discard undesirable recessive alleles by controlling simple traits. Grain yield heterosis was common among varieties, especially in the dent × flint crosses, with mid-parent heterosis values ranging from 18.7% to 32.5%. Dent × flint and dent × flour crosses produced greater yield than dent × dent crosses (Hayes and Olson, 1919; Waldron, 1924). This was not news for ND since it was known that controlled crosses between selected varieties improved grain yield performance (Beal, 1878) and because properly selected crosses could be used for commercial production (Hayes and East, 1911).

The inbred-hybrid concept was developed in the public sector. The desirable consequence was hybrid maize. Hybrid maize traces back to the early 1900s. E. M. East, later replaced by H. K. Hayes and D. F. Jones, led one of the research groups that discovered and revealed the potential of hybrid maize using inbred lines of the variety Leaming. East was directly influenced by the biological principles of Darwin, Mendel (40 years after Imre Festetics in Hungary) and Vilmorin in relation to plant improvement (Hayes, 1956). East related those principles to more practical plant improvement studies to achieve his goals. Probably the most well-known public scientist in early hybrid maize research is G. H. Shull, whose maize research records date back to 1904. At that time research focused on heredity as a basis for improving plants and animals, and Shull studied the theory of genetics and its application to plant breeding. East at Connecticut and Shull at Cold Spring Harbor independently started studies of inbreeding and crossbreeding in 1906 (Hayes, 1963) and provided essential insight into the efforts of maize inbreeding (East, 1908; Shull, 1909). However, Shull discontinued his studies in 1916, since he concluded the inbred-hybrid concept had no practical value due to the small amount of seed produced on inbred lines. Jones suggested to East a procedure that would make hybrid maize a reality for industry and farmers, using the already developed Leaming lines as females and Burr's White inbreds as males (Jones, 1918). Producing seed on a single-cross parent provided a solution to marketing hybrids annually to farmers. After years of intensive and at times discouraging research searching for effective inbred lines, the largest maize research organization in the private sector at that time (Funk farms) produced its first elite maize double-cross hybrid (US13) using lines developed in the public sector (Crabb, 1947). The first

Pioneer hybrid for sale was Copper Cross in 1924 and included East's lines on the female side (Hayes, 1963). These lines were developed from Leaming (Leaming, 1883; Lloyd, 1911). The successful development of the hybrid maize industry was due to cooperation between both the public and private sectors on maize research.

Hybrid production in ND was initiated in the 1930s and the first long-term NDSU official maize breeder, William Wiidakas, joined the ND Agricultural College in 1932 after earning his MS degree from the University of Minnesota under the direction of H. K. Hayes. Therefore, the NDSU program probably had a large influence from East ideas. One decade later, the percentage of maize acreage planted to hybrids was 12%, ranging from 5% in northern and western ND to 35% in southeastern ND by 1941 (Wiidakas, 1942). This percentage increased to 50% by 1948 when successful hybrids (NODAK hybrids) were released, produced by contract growers, and grown extensively in the state (80% in southeastern ND). These were early yellow-dent hybrids ranging from 75 RM to 90 RM (Wiidakas, 1942) and yielded up to 37% more than popular OP varieties. Trials comparing maize OP varieties and hybrids were initiated in Fargo, with the first annual public report made available in 1938. These trials allowed the establishment of several maize maturity zones in the state. Early maturing hybrids were needed for the remainder of the state and popular OP varieties (e.g. Falconer) were still a safe choice for very short-season areas. Inbred lines were developed from local OP varieties, from high-yielding single crosses, and from genetic material obtained in exchanges with land-grant universities. Early maturing lines susceptible to root lodging were backcrossed to late maturing lodging-resistant lines from Minnesota (MN) and Wisconsin (WI) when the exchange of germplasm was at its highest level, a model later followed by Rinke and Sentz (1961) to develop MN lines. As a consequence, useful yellow-dent inbred lines (e.g. ND203, ND230) were developed and utilized by industry as a source of earliness, standability, grain yield and cold tolerance.

H. Z. Cross, the second official NDSU maize breeder (trained by M.S. Zuber of the University of Missouri at Columbia, MO), followed an approach using unique germplasm. New synthetic varieties were developed and improved in several ways. The predominant methods utilized were modified mass selection and modified ear-to-row selection. Limited reciprocal recurrent selection among developed populations was conducted, which proved to be an efficient method to develop new inbred lines in ND. In the 1970s inbred lines were mainly developed from elite × elite crosses using inbreds developed from Wisconsin (WI) and North Dakota (ND). An example of a successful line released in 1980 and utilized by industry is ND246 (Cross, 1980). In the 1980s and 1990s, however, inbred lines were developed using synthetic varieties and other genetically broad-based populations with the purpose of broadening U.S. maize germplasm.

*Present*

North Dakota State University (NDSU) has long supported and continues to support plant breeding. Eleven plant breeding programs are currently housed in the department of Plant Sciences with measurable returns on investment. As a consequence an Applied Plant Breeding Institute (APBI) has been established and genetic improvement as a consequence of plant breeding is the main cause of ND production agriculture generating more than $4.0 billion in cash receipts today.

ND represents a large portion of the increasing northern Corn Belt market. Maize area and productivity have grown rapidly in ND during the past five years, where maize ranks among the top three crops in value with a production of approximately 4 million tonnes (Carena and Ransom, 2006). Continuous genetic improvement of genetically complex traits under extensive testing across ND environments has been essential for the long-term economic success of the maize crop in ND.

The current goal of the maize-breeding program at NDSU (with M. J. Carena as the third official NDSU maize breeder, trained by A. R. Hallauer of Iowa State University at Ames, IA) is to conduct maize breeding research for the northern Corn Belt emphasizing germplasm adaptation, choice and improvement, as well as population and inbred line development. In addition, the NDSU breeding program coordinates the state hybrid maize performance trials, assesses profitable farmer alternatives, and continues to train the next generation of applied maize breeders currently highly demanded by industry.

The NDSU maize program is the only U.S. public program developing very early maturing maize cultivars that actively trains MS and PhD applied maize breeding students. The breeding program works toward releasing inbred lines and populations that are competitive in performance, drying costs (maturity), lodging resistance, drought and cold tolerance, and quality to those of the industry for ND and that could also be used for northern U.S. states and Europe. To enhance project productivity, the breeding program has transitioned from late (S6) to early (S1, S2) and late (S4, S5) generation testing (plus visual selection of early generation lines across three environments), has established a disease nursery, has planted two maize generations per year in several winter and greenhouse nurseries since 1999, and has increased the number of locations to 15 state locations. Major priority is to keep the scientific basis of breeding active in order to separate G×E effects for multi-trait (e.g. use of heritability and rank-summation indices) and multi-stage selection on quantitative traits of economic importance under different genetic backgrounds. Also, major cooperation with several NDSU Departments, maize grower associations, industry partners (e.g. use of commercial testers and biotechnology), national and international institutions, and researchers has been a priority within the breeding program. One good example is our active cooperation with the USDA–GEM project, in which our efforts are the first to incorporate exotic elite tropical germplasm in the northern Corn Belt. In addition, the result of three years of work on stratified mass selection on elite tropical and late temperate germplasm

was tested in 2005. Preliminary data suggest good average progress in earliness (1.3 days $year^{-1}$) and grain moisture at harvest (20 g $kg^{-1}$ $year^{-1}$), while maintaining or even improving grain yield in improved early versions of BSK and BS11. These are new sources for high grain yield and earlier maturity at a very low cost and they are already under testing in hybrid combinations in 2006.

The maize-breeding program at NDSU has released and distributed diverse early maturing inbred lines and populations [e.g. ND291, ND2000, NDSAB(MER-FS)C13] to several private and public institutions that have influenced the early maturity market in the 21st century (Carena and Wanner, 2003; Carena and Cross, 2003; Carena et al., 2003; Carena, 2005a). The common aspects associated with requests from NDSU are early maturity, high grain yield and agronomic performance, high quality, specific traits, and, most important, new unrelated sources of genetic variation. Currently most of our breeding efforts rely on pedigree selection of inbred lines derived from elite by elite combinations. However, testcross trials are complemented with recurrent selection trials of improved populations that serve as a source of new and diverse lines that feed into the pedigree selection program as well. Several thousand early-generation lines have been annually screened for abiotic stresses, especially drought and cold tolerance, in winter and summer nurseries, significantly speeding up pedigree selection for inbred lines. Data from 2005 (more than 15 single-cross trials) have shown significantly highly vigorous NDSU × Industry hybrids with similar grain yield and lodging performance, above average test weight (~3 lb/Bu) and below average grain moisture at harvest (~40 g/kg) compared to dominant commercial corn hybrids available in ND.This might be the major impact for ND producers as a consequence of the continued support for this applied breeding program. Several new sources of valuable traits were identified and as a consequence the NDSAB(MER-FS)C13 elite population was developed, released, and entered in national databases in December 2004. Even though this is a genetically broad-based population there have been several requests for licensing it from the industry as a consequence of the comparative performance of derived inbred lines in hybrid combinations with popular hybrids such as Pioneer 39D82.

The goal of this manuscript was to identify genetically broad-based maize germplasm with impact in early maturing public and private breeding programs that could serve as elite diverse sources of inbred lines and/or improved populations for participatory maize breeding programs.

## Materials and methods

*Choice of germplasm*

Improved synthetic populations were selected for this presentation based on preliminary data. These populations were developed from unrelated sources. Elite improved parents from three different maize-breeding programs [NDSAB(MER)C12, NDSCD(M)C10, NDSM(M)C7, NDSG(M)C15, CGSS(S1-S2)C5, CGL(S1-S2)C5, Leaming(S1-S2)C4, BS5, BS21(R)C7 and BS22(R)C7] were crossed in a diallel mating design, and evaluated with their respective 45 crosses and nine hybrid checks in partially balanced lattice experiments with two replicates at 20 ND environments across years and locations (Carena, 2005b). Analyses of variance (ANOVA) were

performed within and across environments following the Gardner and Eberhart Analysis III model. The top seven populations were selected for a second diallel mating design including reciprocals following Cockerham (1963) statistical analysis, including not only GCA and SCA effects but also maternal and reciprocal effects. Forty-two crosses and eight popular commercial checks were included in a randomized complete block design in western and eastern ND experiments. Data were collected for grain yield (adjusted on a 155 g $kg^{-1}$ grain moisture basis and expressed as t $ha^{-1}$), grain moisture (g $kg^{-1}$), root lodging (%), stalk lodging (%), as well as other agronomic traits. Expected mean squares were calculated based on a mixed linear model that considered replications and environments as random effects and genotypes as fixed effects. Mean comparisons were assessed by the F-protected least significant difference (FLSD), since it provides a suitable mean separation test for an adequate detection of differences (Carmer and Swanson, 1971).

## Results and discussion

This research represents efforts in the NDSU maize-breeding program toward adapting, identifying and improving diverse germplasm, while maximizing the efficiency of pedigree selection for elite and diverse inbred line development. The development of useful maize cultivars requires a good choice of adapted germplasm after extensive testing as well as maximizing the genetic improvement of selected sources of inbred lines. In 1999, a population hybrid program was initiated at NDSU as a consequence of breeding efforts focused on identifying alternative heterotic patterns for the northern Corn Belt. Therefore, the assessment of profitable farmer alternatives was also possible.

Genetically broad-based U.S. temperate maize populations and population hybrids were identified that could have an impact on public and private breeding programs as a consequence of long-term breeding efforts on diverse and useful genetic resources. These serve as good complementary sources for inbred line development along with pedigree selection and for participatory maize breeding programs as well. As a consequence, four reciprocal full-sib recurrent selection programs on selected improved populations are already underway. At least six locations are used for screening each cycle of selection by producing $S_1$ lines before testing.

The results from the first diallel show promising data for certain population hybrids (Table 1). The data also show that maize genetic diversity is ready to be exploited even without the need for reciprocal recurrent selection programs. The challenge is to identify diverse crosses with high mean performance for economically important traits, including grain quality traits and abiotic stresses (Osorno and Carena, 2004; Sezegen and Carena, 2005), and one successful option is through extensive testing of population hybrids.

Even though elite populations have been moderately utilized as improved adapted sources of elite inbred lines for quantitative traits, breeding programs were able to identify elite maize population hybrids with adequate high-parent heterosis with specific genetic effects, as in this example. They could serve both as sources of elite inbred lines and as sources of commercial population hybrids. Moreover, the lack of reciprocal and maternal effects for economically important traits in the second diallel allows the selection of the most productive improved populations for reduced seed production cost.

*Table 1*
Mid-parent (MP) and high-parent (HP) heterosis for grain yield based on good germplasm choice from identified potential maize population hybrids that could serve as alternative heterotic patterns for early maturity regions

| Population, hybrids | Avg. heterosis (%) | | References |
|---|---|---|---|
| | MP | HP | |
| BS10(FR)C8 × BS11(FR)C8* | 39.5 | 34.1 | Eyhérabide and Hallauer (1991) |
| BSSS(R)C11 × BSCB1(R)C11 | 76.0 | 72.4 | Keeratinijakal and Lamkey (1993) |
| BS21(R)C7 × BS22(R)C7 | 45.3 | 43.2 | Carena (2005b) |
| BS21(R)C7 × CGSS($S_1$-$S_2$)C5 | 50.6 | 43.2 | Carena (2005b) |
| BS21(R)C7 × CGL($S_1$-$S_2$)C5 | 55.8 | 52.0 | Carena (2005b) |
| BS22(R)C7 × LEAMING(S)C4 | 43.0 | 36.2 | Carena (2005b) |
| SynB(S)C6 × CCGPB(RRS)C3 | 28.9 | 26.7 | University of Guelph (2002) |
| Average across population hybrids[1] | 48.4 | 44.0 | |
| Average across improved hybrids[2] | 18.8 | 11.1 | Hallauer and Miranda (1988) |

*Cycle 13 of the same reciprocal full-sib selection program was tested and lower values were obtained; [1]Average of population hybrids presented in this table; [2]Average of 71 variety crosses including 25 improved parent varieties

Keeping the scientific basis of plant breeding will be essential for improving quantitative traits across genetic backgrounds. As a consequence, the continuity of recurrent selection programs will be essential for the genetic improvement of useful genetic resources, emphasizing extensive testing to reduce the gap between predicted vs. realized genetic gain. However, good germplasm choice will always depend on how extensive and efficient testing is.

There is the need to keep applied breeding programs strong to link successful germplasm conservation efforts with its utilization and improvement. The exploitation of better hybrid combinations will be essential for maize germplasm utilization as a source of new and diverse inbred lines. Cooperation with the private sector will be needed for long-term research and training as well as to avoid duplications in expensive research (e.g. high throughput genotyping) that quickly becomes obsolete.

A good choice of germplasm based on genetic and statistical principles provides the genetic basis for variation and valid experimental techniques for measuring differences. The extensive testing of population hybrids should increase the use and exploitation of genetic diversity in reserve and the rates of average trait genetic gains in the 21st century. Plant breeding efficiency will increase as scientific research expands our knowledge of the genetic base of plant performance (Fehr, 1991), as long as this knowledge is applied making a large impact. It is still essential to evaluate the performance of crosses with extensive and accurate data collected from observations (Melchinger, 1997; Hallauer, 1999; Cross et al., 2003; Melani and Carena, 2005; Barata and Carena, 2006).

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# QUALITY RESPONSES OF WINTER WHEAT CULTIVARS TO NITROGEN AND FUNGICIDE APPLICATIONS IN CROATIA

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Winter wheat (*Triticum aestivum* L.) cultivars may differ in grain quality responses to nitrogen (N) and fungicide applications, the two most important management inputs in the temperate climates of Europe. Limited information is available on N and fungicide effects on wheat quality in Croatia, where the wheat crop is widely grown under low N inputs without fungicide application. Field experiments were conducted during three years to evaluate the effects of fungicide (tebuconazol applied around heading) and two N fertilization rates on the grain quality of six widely grown winter wheat cultivars. Most cultivars failed to achieve the minimum breadmaking standards at a low N rate because of low protein content (103 g $kg^{-1}$), Zeleny sedimentation (22.2 $cm^3$) and wet gluten (201 g $kg^{-1}$). High N fertilization significantly increased these quality traits in all cultivars (an average of 21% for grain protein, 58% for Zeleny sedimentation and 40% for wet gluten). However, some cultivars with low genetic potential for accumulating grain protein failed to reach the breadmaking quality even at a high N rate when the N availability in the soil was limited by drought. Significant cultivar × N interactions existed for all grain quality traits, but were mainly associated with differences in the magnitude of the responses and less with the ranks. Five cultivars consistently showed increased falling number as the N rate rose, but these cultivar-specific responses to N fertilization were of much lesser magnitude than those across years. Fungicide application showed no effect on grain protein, Zeleny sedimentation, wet gluten or gluten index in all six cultivars tested, whereas one cultivar consistently showed decreased falling number after fungicide use. Only the hectolitre weights increased following fungicide application, especially for disease-susceptible cultivars at the high N rate. Thus, high N fertilization resulted in cultivar-dependent increases in protein content, Zeleny sedimentation, wet gluten and falling number, whereas fungicide application had no effect on grain quality except improved hectolitre weights.

**Key words**: breadmaking quality, falling number, grain protein, gluten index, hectolitre weight, wet gluten, Zeleny sedimentation

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## Introduction

Foliar fungicides and high N fertilization rates are important management inputs to winter wheat production systems in high-yielding areas of Europe. No information is available on the effectiveness of foliar fungicides on the grain quality of wheat in Croatia. The wheat grain yield averaged 3.9 t $ha^{-1}$ nationally (Anonymous, 2002) in the last decade (1991–2001). This relatively low average yield is due to wheat in Croatia being widely grown under low inputs, primarily low N fertilization rates and without fungicide application, despite the high-yielding potentials of current cultivars and the favourable environmental conditions (Varga et al., 2001).

Grain protein content, which is important for good breadmaking quality (particularly loaf volume), can be significantly increased by N fertilization (Szentpétery et al., 2005). High rates of N fertilization can also improve grain quality by increasing sedimentation and wet gluten or resulting in a higher falling number (Webb and Sylvester-Bradley, 1995; Sip et al., 2000; Tanács et al., 2005). However, wheat cultivars may significantly differ in the grain protein response to N fertilization (Bruckner and Morey, 1988; Kelley, 1993), even though a non-significant cultivar × N interaction for grain protein was also reported (Ayoub et al., 1994). Increasing rates of N fertilization may also increase the severity of fungal diseases (especially powdery mildew) in wheat, resulting in decreased hectolitre weight (Roth et al., 1984). Low hectolitre weights are caused by shrivelled grain and are undesirable because of the resulting low flour extraction (Altaf et al., 1969).

Foliarly applied fungicides are widely used to combat foliar diseases in the temperate and maritime climates of Europe. However, grain quality responses to fungicide application in wheat may depend on the severity of specific foliar diseases, cultivar disease resistance, management inputs and environmental conditions. Hectolitre weight, an initial indicator of grain quality, can be significantly increased by appropriate disease control (McKendry et al., 1995; Puppala et al., 1998; Kelley, 2001), even though non-significant responses have also been reported (Herrman et al., 1996). In addition, the effects of fungicide applications on hectolitre weight are cultivar-dependent (Puppala et al., 1998) and influenced by the environmental conditions (Kelley, 1993). Foliar application of fungicides increased the protein content in wheat grain (Herrman et al., 1996; Puppala et al., 1998). In contrast, grain protein was reported in some studies to decrease after fungicide applications (Gooding et al., 1994; Ruske et al., 2003), even though other grain quality parameters were improved (e.g. overall loaf quality). Wheat cultivars may exhibit different magnitudes of grain protein response to fungicide spraying (Karjalainen and Salovaara, 1988; Puppala et al., 1998) or may not show any response at all despite differing in maturity and disease resistance (Kelley, 2001).

The objective of this study was to evaluate the effects of different N fertilization rates and fungicide application on the grain quality responses of six widely grown winter wheat cultivars in Croatia.

## Materials and methods

*Field experiment*

Field experiments in a winter wheat–maize (*Zea mays* L.)–soybean [*Glycine max* (L.) Merr.] crop rotation were conducted in northwestern Croatia in the experimental field of the Faculty of Agriculture, Zagreb during the 1999–2000 (hereafter termed 2000), 2000–2001 (2001), and 2001–2002 (2002) growing seasons on a silt loam soil (Typic Udifluvents). The experimental design was a split-strip design with five replications of each treatment combination. Nitrogen fertilization treatments (high and low) formed ten main plots. The high N fertilization rate involved 194 kg N $ha^{-1}$, of which 86 kg N $ha^{-1}$ was applied before planting. The remainder was topdressed at growth stages (GS) 22 (54 kg N $ha^{-1}$), 24 (27 kg N $ha^{-1}$) and 31 (27 kg N $ha^{-1}$) (Zadoks et al., 1974). The low N fertilization rate consisted of 67 kg N $ha^{-1}$, with 40 kg N $ha^{-1}$ applied before planting followed by one topdressing application of 27 kg N $ha^{-1}$ at GS 24. The subplots consisted of six winter wheat cultivars widely grown in Croatia, differing in their genetic potential for grain protein and susceptibility to foliar diseases. Foliar fungicide applications (+ and –) were striped across the subplots and separated by a 1.5-m border. The fungicide tebuconazol at 250 g a.i. $ha^{-1}$ was applied as a foliar spray in 200 L water $ha^{-1}$ each year. Fungicide application was targeted to GS 55 and sprayed on 13 May 2000, 10 May 2001 and 17 May 2002 in successive seasons.

The crops preceding wheat in the rotation (maize and soybean) were grown under low N rates to minimize the effect of residual nitrogen. In October of each year, 500 kg $ha^{-1}$ N:P:K fertilizer (8:26:26) was broadcast before mouldboard ploughing at 30 cm. Additional urea [$(NH_2)_2CO$] (46% N) at 100 kg $ha^{-1}$ was broadcast before seedbed preparation on plots receiving the high N rate. The cultivars were planted at the recommended rate (770 seeds $m^{-2}$). At seeding the plots consisted of 10 rows 8.0 m long and 0.12 m apart. Wheat was planted on 16 Oct. 1999, 28 Oct. 2000 and 17 Oct. 2001, within the optimum planting-date window for the region. The herbicides amidosulfuron and bromoxynil at 225 g a.i. $ha^{-1}$ were applied at GS 24 to control weeds. Granular N [27% ammonium nitrate ($NH_4NO_3$)] was broadcast by hand in each topdressing application for the high and low N fertilization rates. The harvest dates were 1 July 2000, 4 July 2001 and 3 July 2002.

Visual observations of the flag leaf were made about 2 weeks after the fungicide application to determine the presence and severity of foliar diseases. Cultivar susceptibility to foliar diseases was calculated from the disease severity ratings in 2002, when the highest disease pressure occurred. Based on these ratings, the tested cultivars were referred to as disease-susceptible (Marija, Žitarka, Srpanjka and Soissons) or disease-resistant (Renan and Kuna).

*Laboratory analyses*

Hectolitre weight was determined using standard procedures (Schopper chondrometer). Grain nitrogen was measured by Kjeldahl analysis (ICC 105/2, International Association for Cereal Chemistry, 1994), and protein content was calculated as 5.7 × N content in the dry matter. Zeleny sedimentation and Hagberg falling number were determined using ICC methods 116/1 and 107/1, respectively (International Association for Cereal Chemistry, 1994). Wet gluten and gluten index were measured on a Perten Glutomatic 2200 System according to standard method ICC 155 (International Association for Cereal Chemistry, 1994).

*Statistical analysis*

The experiment consisted of three treatment factors: two N fertilization rates (main plots), six winter wheat cultivars (split-plot) and fungicide application vs. non-treated plots (striped across each block), with five replications. The data were analysed using mixed model procedures (SAS Inst., 1997). Combined analysis of variance was computed with year, N fertilization rate, cultivar and fungicide considered fixed. Mean differences were assessed using the LSD values if the *F*-test was significant at $P = 0.05$. Direct relationships between grain quality traits were analysed with simple Pearson correlation coefficients.

## Results and discussion

*Hectolitre weight*

Hectolitre weight was significantly affected by the year, N fertilization, fungicide, cultivar and their interactions (Table 1). The highest hectolitre weight occurred in 2000 and was significantly larger than that in the two subsequent growing seasons (Table 2). Under low disease occurrence in 2000, the hectolitre weight in untreated plots was higher at the high N fertilization rate than at the low N rate, whereas the opposite pattern of response was observed in the two subsequent growing seasons (Table 2). The decrease in hectolitre weight with high N fertilization in 2001 and 2002 was attributed to the more severe incidence of foliar diseases compared to those found at the low N rate, as also reported by Roth et al. (1984).

Foliar fungicide application tended to improve hectolitre weight in all growing seasons by an average of 1.0 kg $hl^{-1}$ (1.3%). Slightly increased hectolitre weight was found after fungicide treatment in 2000 and 2001, whereas the largest increase occurred in 2002 (Table 2). The largest difference in average 1000-kernel weight was observed between sprayed (40.1 g) and untreated plots (36.2 g), also in 2002. Consequently, a positive correlation ($r = 0.62^*$) existed between hectolitre weight and 1000-kernel weight. A significant year × fungicide × N interaction existed (Table 1), because greater responses to fungicide application at high N than at low N fertilization were more evident in growing seasons with higher disease severity. Thus, following fungicide application in 2002, the increase in hectolitre weight at the high N rate averaged 3.3 kg $hl^{-1}$ compared to a relatively small increment of 1.0 kg $hl^{-1}$ at the low N rate. In contrast, under low disease incidence in 2000, these increments in hectolitre weight following fungicide treatment were similar at both high and low N fertilization rates, averaging 0.2 kg $hl^{-1}$ (Table 2).

Although all the cultivars tended to have increased hectolitre weight following fungicide application, significant differences existed in the magnitude of the responses (Table 1); cultivars susceptible to foliar diseases showed larger increments in hectolitre weight than resistant cultivars. These cultivar-specific responses to fungicide application varied across the N rates and years (Table 1). Similar results were shown by Kelley (2001). In the present study low hectolitre weight was recorded for the disease-susceptible cultivars Marija (71.6 kg $hl^{-1}$)

and Soissons (71.4 kg $hl^{-1}$) in untreated plots at the high N rate in 2002, when disease incidence was severe. After fungicide spraying, these two cultivars improved their hectolitre weight by 7.8% (Marija) and 7.3% (Soissons), whereas the disease-resistant cultivars increased their hectolitre weight by only 2.1% (Renan) and 3.1% (Kuna). At low N fertilization in the same growing season of 2002, the increase in hectolitre weight after fungicide spraying was of lesser magnitude for both susceptible and resistant cultivars because of the lower incidence of foliar diseases in comparison with that observed at the high N rate.

*Table 1*
Analysis of variance for winter wheat quality traits

| Source of variation | df | Hectolitre weight | Protein content | Zeleny sedimentation | Wet gluten content | Gluten index | Falling number |
|---|---|---|---|---|---|---|---|
| Year (Y) | 2 | *** | *** | *** | *** | * | *** |
| R / Y | 12 | — | — | — | — | — | — |
| Nitrogen (N) | 1 | *** | *** | *** | *** | ** | *** |
| Y × N | 2 | *** | *** | *** | * | *** | NS |
| Error a | 12 | — | — | — | — | — | — |
| Cultivar (C) | 5 | *** | *** | *** | *** | *** | *** |
| Y × C | 10 | *** | *** | *** | *** | *** | *** |
| N × C | 5 | *** | *** | *** | ** | NS | *** |
| Y × N × C | 10 | *** | * | *** | *** | *** | * |
| Error b | 120 | — | — | — | — | — | — |
| Fungicide (F) | 1 | *** | NS | NS | NS | NS | NS |
| Y × F | 2 | *** | NS | NS | NS | NS | NS |
| Error c | 12 | — | — | — | — | — | — |
| N × F | 1 | *** | NS | NS | NS | NS | NS |
| Y × N × F | 2 | *** | NS | NS | NS | NS | NS |
| Error d | 12 | — | — | — | — | — | — |
| C × F | 5 | *** | NS | NS | NS | NS | ** |
| Y × C × F | 10 | *** | NS | NS | NS | NS | NS |
| N × C × F | 5 | *** | NS | NS | NS | NS | NS |
| Y × N × C × F | 10 | ** | NS | NS | NS | NS | NS |
| Error e | 120 | — | — | — | — | — | — |

*Table 2*
Average hectolitre weight (kg $ha^{-1}$) of winter wheat cultivars following fungicide application at low (67 kg $ha^{-1}$) and high (194 kg $ha^{-1}$) N fertilization rates (Zagreb, 2000–2002)

| Growing season | Low N rate | | High N rate | |
|---|---|---|---|---|
| | No fungicide | Fungicide | No fungicide | Fungicide |
| 2000 | 81.0 | 81.2 | 81.7 | 81.9 |
| 2001 | 79.2 | 79.3 | 78.4 | 79.5 |
| 2002 | 79.8 | 80.8 | 75.2 | 78.5 |

LSD=0.21 between means within the same growing season and N rate; LSD=0.53 between means within the same growing season and fungicide treatment

*Protein content, Zeleny sedimentation, wet gluten and gluten index*

Grain protein, Zeleny sedimentation, wet gluten and gluten index were significantly affected by year, N rate, cultivar and their interactions (Table 1). The highest protein content was found in 2002 (Table 3). Lower grain yields were produced in 2000 (7,344 kg $ha^{-1}$) and 2001 (6,753 kg $ha^{-1}$) when compared to the yield in the most favourable growing season of 2002 (8,064 kg $ha^{-1}$). The lower yields in 2000 and 2001 were primarily due to the drier than normal weather conditions, which in turn brought about the limitation of N availability in the soil, thus also resulting in decreased grain protein. Consequently, the grain yield was positively correlated with the protein content ($r = 0.59^*$) in this research, as also recently reported by Garrido-Lestache et al. (2004). Zeleny sedimentation and wet gluten responded similarly to protein content because they were significantly greater in 2002 than in 2000 and 2001 (Table 3). Therefore, protein content was positively correlated with Zeleny sedimentation ($r = 0.91^*$) and wet gluten ($r = 0.92^*$), as also found by others (Ćurić et al., 2001; Varga et al., 2003). In contrast, the average gluten index was the highest in 2000, with a small (but significant) decrease in the two subsequent growing seasons (Table 3).

High N fertilization significantly increased grain protein in all growing seasons (Table 3). Across all treatments, protein content averaged 125 g $kg^{-1}$ at the high N rate compared to only 103 g $kg^{-1}$ at low N fertilization, the latter being below the minimum Croatian standard for breadmaking wheat ($\geq$ 115 g $kg^{-1}$). These results demonstrated that high N fertilization appears necessary to provide sufficiently high grain protein content for good breadmaking quality. However, some cultivars with lower genetic potential for high grain protein failed to achieve the minimum quality standard even at the high N rate when drought limited N availability in the soil. Thus, the grain protein content of Srpanjka and Soissons in 2000, and of Soissons in 2001 was below 115 g $kg^{-1}$ (Table 4). At the low N rate, only the two highest protein cultivars, Renan and Kuna, reached satisfactory breadmaking quality under the very favourable growing conditions of 2002 (Table 4).

Although all the cultivars had consistently greater grain protein with the high N rate, the significant cultivar × N rate interaction (Table 1) indicated cultivar differences in the magnitude of the response. Moreover, these cultivar-dependent responses to N fertilization were also affected by the growing conditions (Table 1). Kuna and Renan consistently produced the highest protein content at the high N rate in all growing seasons (Table 4). These two high-protein cultivars exhibited similar trends even at the low N rate, except in 2001 when Marija and Žitarka had significantly higher protein content than Renan and Kuna. However, in the same growing season of 2001, Marija and Žitarka showed a smaller response to high N fertilization when compared to the responses observed for other cultivars and, thus, did not have higher grain protein than Renan or Kuna at the high N rate. A small response to high N fertilization was also observed for Marija in 2000, whereas other cultivars tended to exhibit a similar increase in protein content with high N fertilization in all growing seasons (Table 4).

*Table 3*
Average quality traits of winter wheat cultivars at low (67 kg $ha^{-1}$) and high (194 kg $ha^{-1}$) N fertilization rates (Zagreb, 2000–2002)

| Growing season | Nitrogen rate | Protein content (g $kg^{-1}$) | Zeleny sedimentation ($cm^3$) | Wet gluten content (g $kg^{-1}$) | Gluten index (%) | Falling number (s) |
|---|---|---|---|---|---|---|
| 2000 | Low | 97 | 19.7 | 154 | 95 | 323 |
| | High | 116 | 33.1 | 250 | 91 | 348 |
| 2001 | Low | 101 | 20.5 | 185 | 90 | 259 |
| | High | 120 | 28.6 | 257 | 90 | 289 |
| 2002 | Low | 112 | 26.4 | 263 | 94 | 306 |
| | High | 138 | 43.3 | 340 | 86 | 323 |
| $^{+}LSD_{0.05}$ | | 1.8 | 1.17 | 11.4 | 2.3 | NS |
| $^{++}LSD_{0.05}$ | | 5.0 | 2.98 | 22.2 | 2.6 | |

$^{+}$LSD values for comparing means within the same growing season; $^{++}$LSD values for comparing means across growing seasons; NS: Not significant for year × N interaction at $P = 0.05$

*Table 4*
Average protein content (kg $ha^{-1}$) of winter wheat cultivars at low (67 kg $ha^{-1}$) and high (194 kg $ha^{-1}$) N fertilization rates (Zagreb, 2000–2002)

| Cultivar | 2000 | | 2001 | | 2002 | |
|---|---|---|---|---|---|---|
| | Low N | High N | Low N | High N | Low N | High N |
| Marija | 98 | 108 | 107 | 122 | 106 | 129 |
| Žitarka | 99 | 117 | 98 | 117 | 114 | 139 |
| Srpanjka | 89 | 111 | 108 | 123 | 112 | 140 |
| Soissons | 93 | 111 | 91 | 111 | 104 | 131 |
| Renan | 99 | 122 | 99 | 123 | 120 | 147 |
| Kuna | 103 | 126 | 103 | 125 | 117 | 143 |

LSD=3.5 between means within the same growing season and N fertilization rate; LSD=3.6 between means within the same growing season and cultivar.

Zeleny sedimentation was significantly (58%) higher at the high N rate, averaging 35.0 $cm^3$ compared with 22.2 $cm^3$ at the low N rate, the latter being below the minimum Croatian breadmaking standard (≥ 30 $cm^3$). Wet gluten also showed a large (40%) increase at the high N rate (averaging 282 g $kg^{-1}$) in comparison with only 201 g $kg^{-1}$ at the low N rate. Sip et al. (2000) and Tanács et al. (2005) also reported increasing Zeleny sedimentation and wet gluten with increased N fertilization rates. In contrast, averaged over all treatments, the gluten index at the high N fertilization rate (89%) was slightly, but significantly lower than at the low N rate (93%). However, the significant year × N interaction for gluten index indicated varying responses across growing seasons (Table 1). In 2000 and 2002, the gluten index was significantly decreased at high N compared to low N fertilization, but these differences were not found in 2001 (Table 3). The largest difference occurred in 2002, when the gluten index averaged 86% at the high N rate compared with 94% at the low N rate. Ćurić et al. (2001) demonstrated that a gluten index higher than 75% is satisfactory for

good breadmaking quality. Therefore, this decrease in the gluten index associated with a high N fertilization rate should not have any detrimental effect on the breadmaking quality of wheat.

Cultivar-specific differences for Zeleny sedimentation closely followed the pattern found for protein content. Kuna and Renan, the two cultivars with the highest grain protein, also had the highest Zeleny sedimentation, regardless of N fertilization (Table 5). In contrast, the lowest Zeleny sedimentation was recorded for Soissons and Marija, which also had the lowest protein content. Although all the cultivars had significantly increased Zeleny sedimentation with high N fertilization, the significant cultivar × N interaction (Table 1) indicated differences in the magnitude of the response. This interaction was mainly due to Marija and Žitarka, which had a smaller than average increase in Zeleny sedimentation with high N fertilization, whereas the opposite pattern was observed for Kuna (Table 5). Similarly to protein content and Zeleny sedimentation, Kuna and Renan also had high wet gluten contents at both N rates, but the highest values were recorded for Žitarka, even though this cultivar had significantly lower protein content and Zeleny sedimentation (Table 5). These results indicated that despite the strong positive correlations between protein content, Zeleny sedimentation and wet gluten, some cultivars with lower grain protein might have higher wet gluten content than cultivars with higher protein content and Zeleny sedimentation. There was a significant cultivar × N interaction, mainly due to the specific responses of Marija and Žitarka, which showed a smaller than average increase in wet gluten (Table 5), whereas all other cultivars tended to have similar responses to high N fertilization. The cultivar-dependent responses to N fertilization for Zeleny sedimentation and wet gluten were also affected by the growing conditions (Table 1), but were mainly associated with differences in the magnitude of the responses and less with the ranks (data not shown).

In contrast to Zeleny sedimentation and wet gluten, gluten index was negatively correlated with protein content ($r = -0.34^*$). Therefore, some cultivars with low grain protein had a significantly higher gluten index than those with higher protein content (Table 5). Interestingly, the highest gluten index was determined for Soissons, even though it had the lowest protein content, Zeleny sedimentation and wet gluten. Conversely, the lowest gluten index was found for Žitarka, the cultivar with the highest wet gluten content (Table 5). At high N fertilization, the cultivars had similar gluten index when averaged over three growing seasons (Table 1). However, there was a significant year × cultivar × N interaction, indicating that some cultivars might show specific responses to N fertilization under certain growing conditions.

Fungicide application had no major effect on grain protein content (Table 1). Similar results were reported by Kelley (1993; 2001), whereas many other researchers (Gooding et al., 1994; Herrman et al., 1996; Puppala et al., 1998; Ruske et al., 2003) demonstrated changes in protein content after fungicide treatment. Even in the growing season of 2002, with the most severe disease incidence at the high N rate, grain protein content was unaffected by fungicide

treatment despite an average 15% decrease in the 1000-kernel weight and a consequent 16% reduction in the grain yields in untreated plots compared with sprayed ones. These results clearly demonstrated that the physiological mechanisms regulating carbohydrate accumulation during the grain-filling period in diseased crops led to a similar level of N accumulation in the grain in all the six cultivars tested, regardless of genetic differences in disease resistance and grain protein contents. The Zeleny sedimentation, wet gluten content and gluten index were also unaffected by fungicide treatment (Table 1); in contrast, Peltonen and Karjalainen (1992) found that one cultivar tested in their research displayed an increase in Zeleny sedimentation following fungicide application.

*Hagberg falling number*

The falling number varied significantly across the years (Table 1), with the largest values in the dry growing season of 2000 (Table 3). In a controlled environment, Gooding et al. (2003) reported an increase in falling number when wheat plants were exposed to moisture stress, especially during the grain-filling period. The smallest falling numbers occurred in 2001, most likely due to the heavy rains after physiological maturity, which delayed harvest for several days. The significant year × cultivar interaction (Table 1) indicated cultivar-specific responses across growing seasons. This was mainly due to the specific responses of Žitarka and Kuna. Žitarka exhibited a significantly lower falling number than all other cultivars except Kuna in 2000 and 2001, while it achieved a satisfactory falling number in the growing season of 2002 (Table 6). Kuna was characterized by the lowest falling number in all the growing seasons, particularly in 2001 (103 s), being well below the minimum standard for breadmaking wheat (≥ 250 s). However, Kuna had a falling number above 250 s in 2002 and a value close to the limit (242 s) in 2001.

*Table 5*
Grain quality of winter wheat cultivars at low (67 kg $ha^{-1}$) and high (194 kg $ha^{-1}$) N fertilization rates (Zagreb, 2000–2002)

| Cultivar | Zeleny sedimentation ($cm^3$) | | Wet gluten content (g $kg^{-1}$) | | Gluten index (%) | | Falling number (s) | |
|---|---|---|---|---|---|---|---|---|
| | Low N | High N | Low N | High N | Low N | High N | Low N | High N |
| Marija | 19.6 | 30.2 | 193 | 259 | 93 | 87 | 341 | 330 |
| Žitarka | 23.5 | 33.9 | 224 | 302 | 83 | 79 | 279 | 314 |
| Srpanjka | 22.2 | 36.0 | 195 | 280 | 97 | 93 | 345 | 364 |
| Soissons | 18.4 | 31.4 | 167 | 256 | 99 | 97 | 323 | 345 |
| Renan | 25.2 | 38.7 | 211 | 299 | 97 | 93 | 290 | 353 |
| Kuna | 24.5 | 39.9 | 216 | 296 | 89 | 85 | 197 | 214 |
| $^{+}LSD_{0.05}$ | 1.31 | | 10.0 | | NS | | 12 | |
| $^{++}LSD_{0.05}$ | 1.28 | | 8.9 | | NS | | 12 | |

$^{+}$LSD values for comparing means across two N fertilization rates; $^{++}$LSD values for comparing means within the same N fertilization rate; NS: Not significant for cultivar × N interaction at $P = 0.05$.

*Table 6*
Average falling number (s) of winter wheat cultivars across growing seasons (Zagreb, 2000–2002)

| Cultivar | 2000 | 2001 | 2002 |
|---|---|---|---|
| Marija | 364 | 310 | 332 |
| Žitarka | 319 | 235 | 336 |
| Srpanjka | 376 | 348 | 339 |
| Soissons | 357 | 335 | 310 |
| Renan | 355 | 312 | 298 |
| Kuna | 242 | 103 | 271 |

LSD=14 between means within the same growing season; LSD=16 between means across growing seasons

The significant cultivar × N interaction indicated cultivar-dependent responses to N fertilization (Table 1). This was mainly because all cultivars except Marija tended to have increased falling number with high N fertilization (Table 5). Webb and Sylvester-Bradley (1995) also found that some cultivars did not have a significantly higher falling number under intensive N fertilization.

When averaged over all treatments, fungicide application showed no significant effect on the falling number (Table 1). However, there was a significant cultivar × fungicide interaction. This interaction was primarily due to the specific response of Renan, because the falling number of this cultivar was significantly lower following fungicide treatment (275 s) compared with unsprayed plots (321 s). All the other cultivars showed a similar falling number regardless of foliar disease management. Gooding et al. (1994) and Tanács et al. (2005) also reported a possible decline in falling number following fungicide application. The absence of a cultivar × fungicide × year interaction indicated that this decrease in falling number for Renan after fungicide application was consistent across years regardless of the growing conditions.

## Conclusions

Most cultivars did not reach the minimum breadmaking quality standards at low N fertilization because of low protein content, Zeleny sedimentation and wet gluten. High N compared to low N fertilization significantly improved these quality traits and thus appears necessary for obtaining good breadmaking quality. Significant cultivar × N interactions occurred for all grain quality traits, but these cultivar-dependent responses to high N fertilization were mainly due to differences in the magnitude of the responses and less to the ranks. Fungicide application displayed no major effect on the quality traits of the tested cultivars, except for improved hectolitre weight.

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# COMPETITION, LIGHT QUALITY AND SEEDLING GROWTH OF RUSSIAN WILDRYE GRASS *(Psathyrostachys juncea)*

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The low seedling vigour of Russian wildrye grass (*Psathyrostachys juncea*) (RWR) limits its use. Shading from durum wheat (*Triticum turgidum*) reduced RWR leaf number, tiller number, leaf area and seedling dry weight in a growth room experiment. Treatments with similar shading differed in tiller number and dry weight, which suggested that light quality may have also contributed to these responses. In a second growth room experiment, light intensity (PAR) and red:far-red light ratio (670:730 nm) were altered by coloured plastic filters suspended above seedlings of Russian wildrye, crested wheatgrass (*Agropyron desertorum*) (CWG) and Dahurian wildrye grass (*Elymus dahuricus*) (DWR). Leaf area, tiller number and dry weight of RWR seedlings were reduced by declining red:far-red light ratio while light intensity differences at similar red:far-red ratio did not affect these variables. CWG exhibited similar responses to declining red:far-red light ratio as RWR, except that it exhibited a seedling weight response to light intensity. DWR tiller number was not responsive to low red:far-red light ratio but rather to low light intensity. However, DWR seedling weight, tiller weight and leaf area were responsive to declining red:far-red light ratio. These results indicate that RWR seedlings are sensitive to light quality changes caused by neighbouring plants.

**Key words**: red:far-red light ratio, competition, seedling establishment, tillering, leaf area, dry matter yield

## Introduction

Russian wildrye grass (*Psathyrostachys juncea* Fisch. Nevski) (RWR) is a Eurasian forage grass introduced to North America for summer and fall grazing by beef cattle in the Northern Great Plains region. It has poor seedling vigour, which limits its use on the semiarid prairie (Holt, 1996). Russian wildrye seedlings can require two growing seasons to become fully established (Leyshon and Campbell, 1992) and this delay is an economic cost for beef producers (Holt, 1996). Other species with greater seedling vigour and more reliable

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seedling establishment, such as crested wheatgrass (*Agropyron desertorum* Fisch. ex Link) (CWG) or Dahurian wildrye (*Elymus dahuricus* Turcz. ex Grieseb.) (DWR), are preferred by beef producers despite inferior summer forage quality compared to RWR.

Selection for improved seedling vigour has been fundamental in the development of improved cultivars of RWR, but the main focus has been the development of genetic material with improved emergence from deep seeding (McLeod et al., 2003). However, it was observed that competition from other plants, whether weeds or annual crops (companion crops) seeded with RWR, reduced its seedling growth after emergence by either shading or altered light quality.

Shading reduces the carbon assimilation and growth rate of forage grasses (Dodd et al., 2005). Forage production was limited more by shading than it was by duration of shading treatments in New Zealand. These authors concluded that shading was more limiting to forage production than light quality.

Photomorphogenetic responses of plants are mediated by the phytochrome photoreceptor molecule, which changes its chemical structure when it absorbs light at either 670 (red) or 730 nm (far-red) wavelength (Casal and Smith, 1989). This reversible photochemical reaction at these specific wavelengths allows the detection of light competition above (horizontal plane) or adjacent (perpendicular or lateral plane) to the target plant. Photosynthetic light receptors absorb light energy at 670 nm but transmit energy at 730 nm. In the presence of competitor plants, the target plant experiences a light environment enriched in far-red light and depleted of red light, which results in a low red:far-red light ratio. The ratio of red:far-red light wavelengths is used to describe the changing light quality environment caused by neighbouring competitor plants (Casal et al., 1990).

Vertically oriented green tissues are sensitive to the red:far-red light ratio received from a field of view which is perpendicular to the growth axis (Casal and Smith, 1989). Grass seedlings perceived impending competition through perturbation in the lateral red:far-red environment even before direct shading occurred (Casal and Smith, 1989). This 'early warning' system would provide an adaptive advantage to seedlings in competitive environments whereby they can modify carbon allocation and alter their growth form before direct shading occurs. Phytochrome-mediated responses indicate that plants can detect neighbouring competitors up to a distance of 30 cm away (Smith et al., 1990). A low red:far-red light ratio reduced tillering and shoot dry weight in Italian ryegrass (*Lolium multiflorum* Lam.) and increased leaf blade and leaf sheath elongation (Casal et al., 1990). Tillering of shade-tolerant tall fescue (*Festuca arundinacea* Schreb.) was reduced by low red:far-red light ratio but not by shading (Wherley et al., 2005)

The effect of light quality and intensity has been examined in pasture grasses and legumes with plastic light filters suspended above the plant canopies (Dodd et al., 2005). This technique was successful in altering both the light intensity and red:far-red light ratio for periods up to several months.

The objectives were: 1. to compare RWR seedling growth and development when shaded by a durum wheat competitor in a controlled environment; and 2. to compare seedling growth and development of RWR, crested wheatgrass and Dahurian wildrye under plastic light filters that altered the red:far-red light ratio in a controlled environment.

## Materials and methods

*Experiment I*

An experiment was conducted in two small growth rooms equipped with incandescent and fluorescent lighting. The temperature was 21°C during light and 12°C during dark periods and the photoperiod was 16 h per day. Photosynthetically active radiation (PAR) was measured with a LiCor quantum sensor (Li-Cor Inc., Lincoln, NE, USA) and varied spatially within the room, but averaged 580 moles $m^{-2}$ $s^{-1}$ across the bench.

Plastic plant pots (22 cm diameter) were filled with soil to a depth of 15 cm and then seeded. Sixteen pots were seeded with 25 seeds of Russian wildrye, cultivar Swift (Alderson and Sharp, 1994) and 64 pots were seeded with 25 seeds of RWR and 10 seeds of durum wheat (*Triticum turgidum* L.) cultivar Kyle. The seeds were covered with 1 cm of additional soil and then all pots were watered to field capacity. At one week after the emergence of wheat and RWR, seedlings were removed as necessary to create the following treatments: 1 RWR seedling + 4 wheat seedlings; 2 RWR + 3 wheat seedlings; 3 RWR + 2 wheat seedlings; 4 RWR + 1 wheat seedling; and 5 RWR seedlings with zero wheat seedlings. Typical field seeding rates would be 60 kg $ha^{-1}$ for wheat and 3 kg $ha^{-1}$ for RWR or the equivalent of 2 and 1 seedlings per pot, respectively. However, the aim was to create a sequential series of light competitive environments for the RWR seedlings, not to mimic field conditions. There were 8 pots of each treatment randomly assigned to each growth room. The pots were watered daily and fertilized weekly with a complete nutrient solution. They were rotated weekly among the positions on the bench in each growth room to compensate for the light intensity gradient from the centre to the edge. Light intensity was determined with a Li-Cor quantum sensor at the top of the wheat canopy and at the top of the RWR seedlings and the proportion of shading determined at 14, 21, 28, 42 and 56 days after planting (DAP). Leaf number and leaf area were recorded on the RWR seedlings at 14, 21, 28, 42 and 56 DAP. Tiller number per RWR seedling was counted at 28, 42 and 56 DAP. At 56 DAP, RWR seedling growth above the soil was harvested, dried at 60 °C, and dry weight recorded. Mean leaf number, tiller number and leaf area at all sampling times and dry weight per seedling at 56 DAP were calculated for each experimental unit (pot) and subjected to analysis of variance with JMP software (V. 3.1. SAS Institute Inc., Cary, NC, USA, 1995). Where treatments were different at $P<0.05$, Fisher's Protected LSD was calculated to separate treatment means.

*Experiment II*

Seeds of RWR cultivar Swift, crested wheatgrass cultivar Nordan, and Dahurian wildrye cultivar Arthur (Alderson and Sharp, 1994) were germinated on moist filter paper in petri dishes and the seedlings were transplanted to 1 L milk cartons converted to pots by removing the top 20 mm and cutting two drainage holes in the bottom. Each pot was filled to within 15 mm of the top with a loam soil mix containing 10% peat moss. The pots were watered daily and fertilized weekly with a complete nutrient solution.

Plastic theatrical colour filters were obtained from Roscolux (1271 Denison St. #66, Markham, ON L3R 4B5). Based on the spectral data provided by the manufacturer, the colours, light transmission and red:far-red light ratios were: #26 Light Red, 12% and 1.26; #61 Mist Blue, 66% and 0.95, #63 Pale Blue, 56% and 0.71; #66 Cool Blue, 62% and 0.34; #68 Sky Blue, 14% and 0.54; and #129 Sky Blue Silk, 14% and 0.38, respectively. Two control treatments, one per growth room, had no light filter between the plants and the lights. The filters were secured in 60 × 120 cm aluminium frames and suspended below the lights and above the pots on the growth room bench. Reflective aluminium foil was suspended vertically between the frames to ensure that light

intensity and red:far-red ratio within any treatment was not affected by the neighbouring treatment(s). The pots were arranged in 4 rows of 11 pots beneath each filter. The seedlings used for counts and measurements were grown in the centre 2 rows of pots. The grass species were randomized among groups of 3 pots (9 in total across the growth room bench). Thus, 18 pots under each filter were used for measurements and counts.

At 7-day intervals for 4 weeks after planting, red:far-red light wavelength ratios were determined with a LI-1800 scanning monochromometer (Li-Cor Inc., Lincoln, NE, USA) fitted with a cosine-corrected sensor at the end of a flexible quartz fibre-optic light guide. The ratio was calculated by dividing light energy at 670 ± 2 nm by the energy at 730 ± 2 nm measured horizontally above the seedlings to measure radiation at the top of the plant canopy, and vertically amidst the seedlings in the centre of each group of six seedlings to measure radiation entering from the horizontal direction. The vertical or lateral light ratio measurement represents the phytochrome-mediated detection of neighbouring plant competition (Ballare et al., 1997). At the same time, the light intensity of PAR was determined with a LiCor quantum sensor. Tiller and leaf counts were made on each seedling. Leaf area per leaf was estimated from leaf length and width according to the formula: Area = (length × width × 0.75). In addition, at 28 days after planting all seedlings were harvested at the soil level and aboveground dry weight determined per seedling. Derived variables included leaf area per leaf and dry weight per tiller.

Analysis of variance based on a randomized block design (Steel et al., 1997) was done with JMP software (V. 3.1. SAS Institute Inc., Cary, NC, USA, 1995) and least-square means were calculated for variables with an unbalanced observation number per treatment. Light filters were grouped based on light ratio and light intensity similarities and group means were compared by contrasts in JMP. Graphics were prepared with SigmaPlot 2000 for Windows software (SPSS Inc. Chicago, IL, USA) but standard errors were calculated in JMP.

## Results

### *Experiment I*

Shading increased over time with the growth of the wheat plants and this result was consistent until 56 DAP (Fig. 1A). The wheat competitors reduced RWR leaf area (Fig. 1B), tiller number (Fig. 1C) and leaf number (Fig. 1D). The effect of shading on these variables increased with each sampling date. Russian wildrye seedling weight at 56 DAP was 0.49 g seedling$^{-1}$ for the control (no competitor) but was reduced by 55% by two wheat competitors and by 67% by four wheat competitors (insert in Fig. 1C). Seedling dry weight was correlated with tiller number ($r = 0.99$, $P<0.01$, $n=5$) leaf number ($r = 0.98$, $P<0.01$, $n=5$), leaf area ($r = 0.98$, $P<0.01$, $n=5$) and shade ($r = -0.93$, $P<0.01$, $n=5$). Seedling dry weight is frequently used to evaluate the seedling vigour of Russian wildrye grass (Jefferson, 1993; Berdahl and Ries, 1997). Seedling dry weight and its components, such as tiller and leaf number per seedling, were reduced by shading in controlled environments.

There was a similar proportion of shading between the 3 RWR & 2 wheat and the 1 RWR & 4 wheat treatments at 56 DAP. However, these treatments differed in tiller number per seedling, leaf area per seedling, leaf number per seedling and seedling dry weight at this date. This suggests that a factor other than shading had contributed to the reduced growth of the RWR seedlings. We were unable to measure the red:far-red light environment in this experiment but it is known that as plant canopies develop, competitor plants and even adjacent seedlings in a cohort will reduce the light quality and cause changes to seedling growth and development (Dodd et al., 2005).

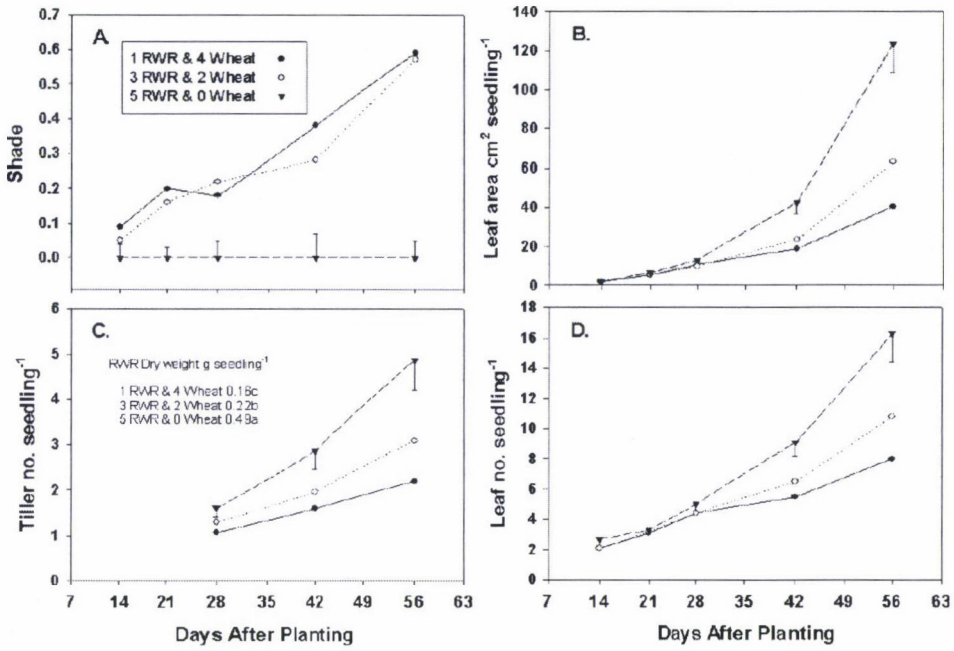



*Fig. 1.* Mean shade (A), leaf area (B), tiller number (C) and leaf number (D) for 1, 3 or 5 Russian wildrye (RWR) seedlings grown with 4 wheat plants, 2 wheat plants or 0 wheat plants, respectively, in pots in a growth room. Treatments with 2 or 4 RWR seedlings with 3 or 1 wheat plants, respectively, are not shown for clarity. Vertical bars represent Fisher's Protected LSD ($P<0.05$) values. RWR seedling dry weight at 56 days after planting is shown as insert in C and dry weight means followed by different letters are significantly different when tested with LSD

*Experiment II*

Plastic theatrical light filters reduced the light intensity and horizontal red:far-red light ratio at 28 DAP compared to the controls, but not as consistently as would be predicted from the manufacturer's specifications (Table 1). Filters # 26 Light Red, #68 Sky Blue and #129 Sky Blue Silk had horizontal red:far-red ratios similar to those predicted. However, filters #61 Mist Blue, #63 Pale Blue and # 66 Cool Blue exhibited higher horizontal red:far-red ratios than predicted. The light transmission specifications provided by the manufacturer were consistent with the observations, as filters #129 Sky Blue Silk, #68 Sky Blue and #26 Light Red reduced PAR more than filters #63 Pale Blue, #66 Cool Blue and #61 Mist Blue. Filters #68 Sky Blue and #129 Sky Blue Silk reduced the horizontal red:far-red light ratio more than the other four filters, which reduced the light ratio compared to the controls.

The lateral red:far-red ratio declined with DAP for the controls and for #26 Light Red, #61 Mist Blue, #63 Pale Blue and #66 Cool Blue (Fig. 2). This was expected as the plants within each filter treatment grew and began to alter

*Table 1*

Photosynthetically active radiation (PAR), red:far-red light ratios, and treatment group designation for 6 plastic light filters and two control treatments at 28 days after planting in a growth room trial

| Light filter | PAR $\mu$moles $m^{-2}$ $s^{-1}$ | Red:far-red light ratio | | Group designation[1] |
|---|---|---|---|---|
| | | Horizontal | Lateral | |
| #26 Light Red | 107 | 1.20 | 0.58 | LM |
| #61 Mist Blue | 433 | 1.32 | 0.57 | HH |
| #63 Pale Blue | 353 | 1.22 | 0.50 | HM |
| #66 Cool Blue | 367 | 0.94 | 0.57 | HM |
| #68 Sky Blue | 87 | 0.46 | 0.36 | LL |
| #129 Sky Blue Silk | 67 | 0.47 | 0.40 | LL |
| Control 1 | 587 | 1.38 | 0.71 | HH |
| Control 2 | 580 | 1.43 | 0.76 | HH |
| $LSD_{0.05}$ | 10 | 0.02 | 0.02 | |

[1]HH high light intensity, high lateral red:far-red light ratio; HM high light intensity, moderate lateral red:far-red light ratio; LM low light intensity, moderate lateral red:far-red light ratio; LL low light intensity, low lateral red:far-red light ratio.

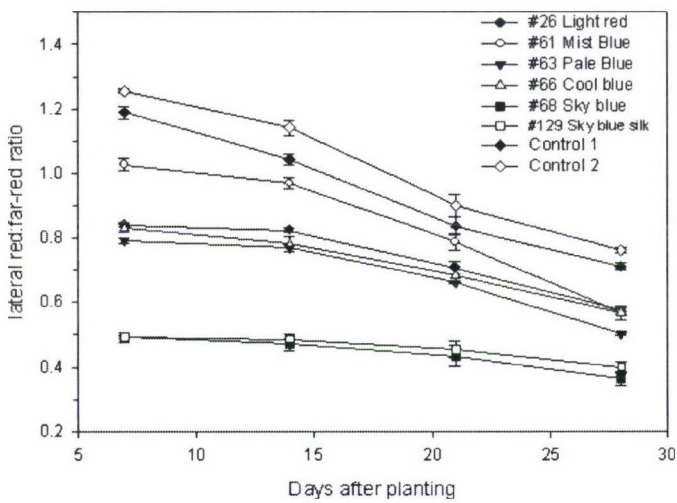



*Fig. 2.* Lateral red:far-red light ratio in growth chambers for grass seedlings under six plastic light filters and two control treatments at four sampling times after planting. Standard error bars are shown for each point

the red:far-red light environment through mutual shading of the target plants and adjacent border plants. The lateral red:far-red ratio was low and similar across DAP for filters #68 Sky Blue and #129 Sky Blue Silk. Three groups of treatments are evident in the lateral red:far-red responses shown in Figure 3. High red:far-red ratio was exhibited by Control 1, Control 2 and filter #61 Mist Blue, moderate red:far-red ratio was exhibited by #26 Light Red, #63 Pale Blue

and #66 Cool Blue, and low red:far-red ratio was exhibited by #68 Sky Blue and #129 Sky Blue Silk. By combining these lateral red:far-red light ratio groups with the light intensity (PAR) readings observed for these six filters and two control treatments, the following four treatment groups were established: high intensity, high red:far-red light ratio (HH) from Control 1, Control 2 and #61 Mist Blue filter; high intensity, moderate red:far-red light ratio (HM) from #63 Pale Blue and #66 Cool Blue filters; low intensity, moderate red:far-red light ratio (LM) from #26 Light Red filter; and low intensity, low red:far-red light ratio (LL) from #68 Sky Blue and #129 Sky Blue Silk. The lateral red:far-red ratios of these four groups over 28 DAP are shown in Figure 3. The HH group has the highest lateral red:far-red light ratio, the HM and LM groups have a moderate red:far-red light ratio, and the LL group has the lowest red:far-red light ratio at all four sampling dates (Fig. 3). Averaged over all dates, the HH group has a PAR of 496 ±84 μmoles $m^{-2}$ $s^{-1}$ and a lateral red:far-red ratio of 0.93±0.20, the HM group has a PAR of 331±30 μmoles $m^{-2}$ $s^{-1}$ and a lateral red:far-red ratio of 0.70±0.11, the LM group has a PAR of 105±6 μmoles $m^{-2}$ $s^{-1}$ and a lateral red:far-red ratio of 0.74±0.11 and the LL group has a PAR of 79±10 μmoles $m^{-2}$ $s^{-1}$ and a lateral red:far-red ratio of 0.47±0.05. The contrast of HH vs HM allows us to examine the impact of lower red:far-red light ratio at nearly full light intensity for growth room conditions, while the contrast of LM vs LL allows us to examine the impact of lower red:far-red light ratio at low light intensity. The contrast of HM vs LM allows us to examine the impact of lower light intensity at similar red:far-red light ratio.

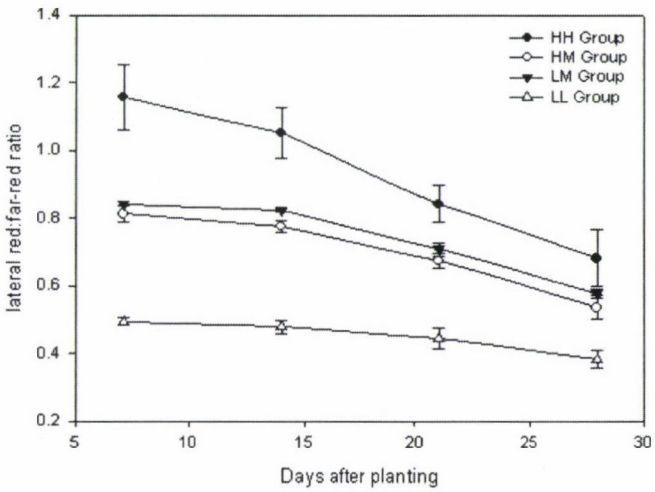



*Fig. 3.* Lateral red:far-red light ratio for four groups of light treatments designated as high intensity and high red:far-red ratio (HH) (mean of Control 1, Control 2 and #61 Mist Blue), high intensity and moderate red:far-red ratio (HM) (mean of #63 Pale Blue and #66 Cool Blue), low intensity and moderate red:far-red ratio (LM) (#26 Light Red) and low intensity and low red:far-red ratio (LL) (mean of #68 Sky Blue and #129 Sky Blue Silk). Standard errors are shown for each point

The group × grass interaction was significant (P=0.02) for tiller number per seedling (Table 2). The interaction was studied by examining the contrasts described above within each grass species. The decline in red:far-red light ratio at high light intensity (HH vs HM) produced a significant decline in tiller number for CWG and RWR but not for DWR (Table 2). A further decline in red:far-red light ratio at low light intensity (LM vs LL) did not result in any further decline in tiller number per seedling for any one of the three grasses. The decline in light intensity at moderate red:far-red ratio (HM vs LM) produced a significant decline in DWR tiller number per seedling but had no effect on the tiller number of crested wheatgrass or RWR. These results indicate that as the lateral red:far-red ratio declined from 0.93 to 0.70, tiller number per seedling is reduced by crested wheatgrass and RWR, while the tiller number of DWR was not affected until light intensity declined. Crested wheatgrass and RWR share a similar tillering response to signals from adjacent neighbour plants and reduce the number of tillers produced. As RWR exhibits a lower number of tillers per seedling than crested wheatgrass (Jefferson, 1993), the proportional effect is larger (47% for RWR vs 27% for CWG). Tillering in DWR is affected by direct shading but not by light quality.

The group × grass interaction was significant (P=0.04) for dry weight per seedling (Table 2). All three species exhibited a reduction in dry weight as the red:far-red ratio declined at high light intensity (HH vs HM). The proportion of decline in dry weight was greatest for RWR (57%) and least for crested wheatgrass (34%). At low light intensity, there was no significant effect of a further drop in red:far-red light ratio (LM vs LL) for CWG or RWR. The effect was nearly significant (P=0.06) for DWR. Dahurian wildrye grass did not reduce tiller number in response to declining lateral red:far-red light ratio, but it did reduce its growth rate, as evidenced by dry weight per seedling. As light intensity decreased at a moderate red:far-red light ratio (HM vs LM), crested wheatgrass significantly reduced seedling dry weight, while DWR and RWR were not affected. These data suggest that the size of tillers must have differed among these three grasses for this pattern of tillering and dry weight differences to have occurred.

The group × grass interaction was significant (P=0.04) for dry weight per tiller (Table 2). Dahurian wildrye tiller size decreased with declining red:far-red ratio at both high intensity (HH vs HM) and low light intensity (LM vs LL). Crested wheatgrass and RWR tiller size were not affected by decreasing red:far-red light ratio at either high light intensity or low light intensity. Crested wheatgrass and DWR tiller size decreased due to decreasing light intensity at moderate red:far-red light ratio (HM vs LM), while CWG and RWR reduced tiller number and dry weight.

The group × grass interaction was nearly significant (P=0.08) for leaf area per leaf, so it was deemed worthwhile to examine the interaction with contrasts within each species (Table 2). The group differences did not affect the leaf size

of CWG. For DWR and RWR, however, a declining lateral red:far-red light ratio caused a significant reduction in leaf area per leaf at both high light intensity (HH vs HM) and low light intensity (LM vs LL). As light intensity decreased at moderate red:far-red ratio (HM vs LM), however, the area per leaf increased for DWR and was unaffected for CWG or RWR.

*Table 2*

Effect of light treatment group (HH - high intensity, high lateral red:far-red ratio; HM - high intensity, moderate lateral red:far-red ratio; LM - low intensity, moderate lateral red:far-red ratio; and LL - low intensity, low lateral red:far-red ratio) and grass species (CWG - crested wheatgrass; DWR - Dahurian wildrye grass; and RWR - Russian wildrye grass) on tiller number per seedling, dry weight per seedling and leaf area per leaf at 28 days after planting. The probability of the grass × group interaction and probabilities for treatment group contrasts are also shown

| Group | Tiller number seedling$^{-1}$ | | | Dry weight g seedling$^{-1}$ | | | Dry weight g tiller$^{-1}$ | | | Leaf area cm$^2$ leaf$^{-1}$ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CWG | DWR | RWR | CWG | DWR | RWR | CWG | DWR | RWR | CWG | DWR | RWR |
| HH | 7.5 | 9.5 | 6.2 | 1.08 | 0.99 | 0.60 | 0.16 | 0.11 | 0.10 | 5.2 | 6.4 | 5.0 |
| HM | 5.5 | 9.3 | 3.3 | 0.71 | 0.61 | 0.26 | 0.13 | 0.07 | 0.08 | 4.7 | 5.1 | 3.9 |
| LM | 4.0 | 4.8 | 3.0 | 0.34 | 0.48 | 0.18 | 0.08 | 0.10 | 0.06 | 4.1 | 7.2 | 5.0 |
| LL | 4.5 | 5.0 | 3.0 | 0.25 | 0.27 | 0.12 | 0.07 | 0.06 | 0.04 | 4.2 | 5.6 | 3.3 |
| Interaction *P* | 0.02 | | | 0.04 | | | 0.04 | | | 0.08 | | |
| Contrast *P* | | | | | | | | | | | | |
| HH vs HM | 0.01 | 0.82 | <0.01 | <0.01 | <0.01 | <0.01 | 0.06 | 0.01 | 0.17 | 0.27 | 0.01 | 0.03 |
| LM vs LL | 0.62 | 0.87 | 0.99 | 0.41 | 0.06 | 0.60 | 0.67 | 0.02 | 0.36 | 0.82 | 0.01 | 0.01 |
| HM vs LM | 0.33 | <0.01 | 0.74 | <0.01 | 0.25 | 0.45 | 0.02 | 0.05 | 0.32 | 0.33 | <0.01 | 0.11 |

## Discussion

Similar tillering responses to low red:far-red light ratio have been observed in wheat (Barnes and Bugbee, 1991), barley (Skinner and Simmons, 1993) and Italian ryegrass (Casal et al., 1987). In wheat, tiller emergence essentially ceased at a red:far-red ratio below 0.35 to 0.40 (Evers et al., 2006). The largest decline in RWR tiller emergence in the present experiment occurred at a red:far-red ratio of 0.70 with little additional decline in tillering due to decreasing light intensity or to decreasing the red:far-red light ratio to 0.45 (LL). In order to adapt to light competition from adjacent plants, RWR reduced its tillering by 47% while CWG reduced its tillering by 27%. A sensitivity to declining red:far-red light environment allows RWR to avoid tillering until additional red light becomes available through disturbance, senescence or some other mechanism that opens the canopy or reduces the presence of neighbour plants and allows more light energy to penetrate to the RWR seedling.

Barnes and Bugbee (1991) reported that tillering in wheat responded to light intensity and light quality in controlled environments. When the red:far-red light ratios declined while light intensity was held constant, tiller number per plant declined. When light intensity was reduced through shading while the

red:far-red light ratio was held constant, tiller number was also reduced. The rate of phenological development was increased in wheat at lower red:far-red ratios. These conclusions are consistent with observations made for CWG compared to RWR. When seeded in light competitive conditions, CWG will reduce tiller production but will accelerate the reproductive development of the main tiller and elevate a seed head and flag leaf tissues above the competing canopy through stem elongation. In contrast, RWR did not initiate stem elongation under the same conditions. It exhibits a distinct juvenile seedling stage (Schaaf, 1961) where reproductive development and stem elongation cannot be triggered by environmental signals. While the phenological development of the grass seedlings was not measured in these trials, main tiller elongation was observed in crested wheatgrass plants under low red:far-red light, while all the RWR plants remained at the vegetative growth stage.

These data suggest that RWR and DWR both reduce leaf size in response to declining red:far-red ratios, but DWR conversely increases leaf size in response to shading. Larger leaf area per leaf can compensate, at least partially, for a reduction in tiller and leaf number per seedling resulting from lower red:far-red light ratio conditions. Larger leaves can be advantageous in capturing sun flecks or intermittent light radiation and maximizing photosynthesis if specific leaf weight (mass per unit leaf area) is reduced. Unfortunately, leaf lamina weight was not measured, so it was not possible to determine whether specific leaf weight was reduced as DWR leaf area per leaf increased under shaded conditions.

From these two experiments, the following generalizations can be made about the response of these species to shading and light competition. Russian wildrye is responsive to declining red:far-red light ratio caused by neighbouring competitor plants. As the lateral red:far-red light ratio declined from 0.9 to 0.7, RWR reduced tiller number, leaf area per plant and leaf area per leaf and ultimately exhibited reduced growth. Light intensity appears to be less important than light quality in signalling the onset of these changes in RWR. In contrast, DWR tillering did not respond to declining red:far-red light ratio but rather to decreasing light intensity. Declining red:far-red light ratio did result in smaller DWR plants, however, and smaller tillers combined with smaller leaves. Shading may cause DWR to increase leaf area per leaf in order to capture as much sunfleck energy as possible. Crested wheatgrass tillering declined, as in RWR, with the first decline in lateral red:far-red light ratio from 0.9 to 0.7, but not as light intensity declined. Unlike RWR, however, CWG growth was reduced by both declining light quality and shading, and did not exhibit any response in leaf size.

Each grass species exhibited different responses to changing light environments and these responses are helpful to explain the agronomic performance in seedling establishment. Dahurian wildrye is known as a species with high seedling vigour that is easy to establish (Lawrence and Ratzlaff,

1985). This can be attributed to its tiller insensitivity to light competitive environments and its ability to alter tiller size and leaf size in response to competition. Crested wheatgrass is known for good seedling vigour and its ability to establish seedlings even under light competitive conditions. This can be attributed to its sensitivity to light quality and shading and its ability to elongate a stem on the main seedling tiller and elevate some photosynthetically active tissues above that of its competitors. Russian wildrye grass is sensitive to declining light quality. When shaded by competitors or exposed to low red:far-red light ratio due to neighbour plants, the RWR tillering rate is more negatively affected than that of CWG or DWR. Tillering is highly correlated with leaf number, leaf area and seedling dry weight in RWR, and all these parameters were negatively affected by low red:far-red light ratio.

Seedling establishment should be managed in field situations to avoid or minimize the negative effect of light competition on RWR seedlings. For example, the use of companion crops to reduce weeds, hold erodible soil against wind erosion, or produce an economic return during the establishment year should be avoided due to competition and light quality effects on seedling growth. Weed control through herbicide application or tillage should be practised so that competition is minimized even where there are generally open canopies. This recommendation may be impossible to implement in large-scale reclamation and/or restoration seedings on grasslands, due to either topographic and edaphic limitations to on-site equipment use or to the fact that herbicide use is not compatible with the project objectives. In such scenarios, we would question whether the choice of RWR is appropriate for such re-seeding projects. If low input management and immediate soil stabilization or protection from erosion are the primary objectives of a grasslands re-seeding project, then species with a more aggressive seedling growth pattern than RWR would be more suitable.

These results will be useful to forage plant breeders. The selection for seedling vigor in the North American RWR breeding programs has focussed on germination and emergence growth stages (Berdahl and Ries, 1997; McLeod et al., 2003). Breeders could exploit variation in seedling tillering rate in RWR under low red:far-red light ratio conditions, if such genetic variation exists, and then select lines with improved tillering, seedling dry weight and seedling establishment. Such selections could improve seedling establishment under field conditions where weed control is uneconomic or companion crops must be used to control soil erosion during forage seedling establishment. Further research is also necessary to confirm our findings under field conditions where light quality and intensity vary and where light competition increases from seedling emergence to the end of the growing season.

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# EFFECT OF 1-MCP TREATMENT ON APPLE BIOCHEMICAL CONTENT AND PHYSIOLOGICAL DISORDERS

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The research was conducted with the apple (*Malus domestica*) cultivars Krameri Tuviõun and Talvenauding at the Estonian Agricultural University during 2003–2005. The aim was to study the effect of 1-methylcyclopropene (1-MCP) treatment (1 ppm, at room temperature, 24 h) on the biochemical content and physiological disorders of apples and on external quality after 4 months of storage. The apples were stored in a normal atmosphere at 2–5°C and 80–85% RH. In the first storage season 1-MCP treatment did not improve apple quality in general; only the peel background colour of treated apples remained greener. In the second storage season 1-MCP treatment influenced the biochemical content of the apples and improved apple quality: treated fruits were firmer (increase from 4.7 kg/cm$^2$ to 8.1 kg/cm$^2$) and contained more vitamin C; also, the SSC decreased and the loss of titratable acids was delayed. Consequently fruit spoilage decreased by 30%.

**Key words:** 1-methylcyclopropene, storage, firmness, vitamin C, soluble solids, titratable acids

## Introduction

The consumption of fresh fruit and vegetables is expected to increase in future due to increasing interest in a healthy lifestyle. In most industrialized countries, cardiovascular disease and cancer are ranked as the top two leading causes of death. The causes of both diseases have been linked to lifestyle choices, and one of the most important is diet (Boyer and Liu, 2004). At the same time, there is increasing concern about the environment. Therefore, together with high-quality products, consumers need to be given information so that they can make environmentally informed shopping choices (Wallen et al., 2004).

Apples are a significant source of beneficial phytochemicals contributing to health, especially in the northern hemisphere. Although several means for improving apple quality and storability have been developed, there is still a need for more effective or more sustainable methods. For example, diphenylamine,

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which is widely used to prevent the post-harvest deterioration of apples, is a compound from the third European Union list of priority pollutants (Drzyzga, 2003). This compound will most likely have to be replaced in future with one that is not an environmental hazard. One alternative could be 1-methylcyclopropene (1-MCP). The safety, toxicity and environmental profiles of 1-MCP with regard to humans, animals and the environment are favourable. The compound is used at low rates, has a non-toxic mode of action and is chemically similar to naturally occurring substances (Blankenship and Dole, 2003). 1-MCP blocks ethylene receptors and prevents ethylene effects in plant tissues: it was found to slow softening and decrease ethylene production; it has also reduced the incidence of superficial scald and other physiological disorders of apples (Watkins et al., 2000). However, as with most of the currently used treatments, its effect is not universal and depends on several factors, such as concentration, treatment time, air temperature and type of fruit (Pre-Aymard et al., 2003). The relationship between ethylene, titratable acids and soluble solids concentration in fruits is not yet clear (Watkins et al., 2000). Similarly there is a lack of information concerning the effect of 1-MCP on beneficial phytochemicals in fruits and vegetables, such as vitamin C.

The aim of the present research was to study the effect of 1-MCP treatment on the biochemical content, physiological disorders and external quality of Talvenauding and Krameri Tuviõun apples.

## Materials and methods

*Plant material*

The experiment commenced in September 2003 and finished in January 2005. In the first year the apple cultivars Krameri Tuviõun and Talvenauding were used for the experiment. The apple trees were grown in a commercial orchard belonging to the Vasula Aed Company, which is situated in Tartu County, South Estonia (58°28'N and 26°44'E). The plantation was 27 years old; rejuvenation was carried out in 1997 and routine thinning has been carried out every year. The apple trees were planted with a distance of about 8 m between rows and 4 m within the row. In the second experimental year, the Vasula apple orchard produced no yield, since late spring frosts damaged the flowers. Therefore, the second year of the experiment was carried out at the Rõhu Testing Centre of the Agricultural Research Centre in South Estonia with the cultivar Talvenauding. The plantation was 18 years old; rejuvenation was carried out in 1999 and routine thinning had been carried out every year. The apple trees were planted with a distance of about 6 m between rows and 4 m within the row. No irrigation system was used in either of the plantations and Antonovka was used as seedling rootstock for both the cultivars. The apples were harvested during the third week of September in 2003 and the last week of September in the second experimental year. Samples of 300 fruits per treatment (50 fruits in six replications) were collected. First grade apples were picked according to an equatorial pattern (North-South-East-West) from the outside of the trees avoiding fruit situated at the top, bottom and deep inside the canopy.

*Weather*

In summer 2003 the air temperature in May, August and September was approximately the same as the average of many years; June was warmer and July cooler than the average of many years (Table 1). At the same time, the summer was very rainy, especially during May, July and August. However, before harvest in September, the weather was relatively dry.

In 2004 spring frosts occurred in May at the time of apple flowering and caused serious frost damage. The air temperature remained lower than the average until July. The second part of the summer and the beginning of autumn was warmer than average. The amount of precipitation fluctuated, being relatively low in May, more than double the average in June, at the average level in July and higher than average in August.

*1-MCP treatment and storage conditions*

For 1-MCP treatment, the volume of apples was calculated and the apples were placed in a 120 l plastic jar. A small electric fan was placed in the jar to mix the air. A concentration of 1 ppm 1-MCP was calculated for the jar spare space and 183 mg of EthylBloc (Rohm and Haas, Philadelphia, with a.i. of 0.14%) was weighed out into a capped test tube. Just before treatment, 4 ml of 40°C water was added to the test tube and the open tube was placed in the jar with the apples. The jar was immediately sealed. The apples were treated for 24 h at room temperature (20°C). After removal the apples were stored in the commercial coolstore of the Vasula Aed Company at 2–5°C and 80–85% RH. Fifty fruits per layer were placed in air-permeable plastic boxes. The storage period was 4 months.

*Measurements and analyses*

Spoiled apples were separated from the healthy apples monthly and divided into categories, namely Talvenauding fruits with superficial scald, rotting and other quality loss symptoms and Krameri Tuviõun fruits with calcium deficiency symptoms (bitter pit), physiological spot, rotting and other. Measurements on dry matter (DM) content as a % (at 105°C), titratable acids (0.1 N NaOH) expressed as % malic acid, water-soluble solids (°brix, digital refractometer ATAGO Co. Ltd., Japan), fruit firmness (Fruit Pressure Tester Effegi FT327, equipped with 11 mm plunger, expressed as kg) and vitamin C content were made on healthy apples after harvest and after four months of storage. Ten apples per replicate were used for each measurement.

For the determination of vitamin C, 10 g of crushed fruit was taken for analysis. As described by Paim and Reis (2000), 60 ml of a metaphosphoric/acetic acid mixture (3% $HPO_3$ + 8% $CH_3COOH$) was added instantly to avoid vitamin C breakdown in air, and 125 ml plastic bottles with leakproof screwcaps were used to shake the dilutions for eight hours using a shaker at 190–200 rpm $min^{-1}$. After that, 2 ml of a starch indicator was added and the solution was immediately titrated with a standard solution of iodine $I_2$ 0.01 g eqv $l^{-1}$ until the solution had lost the initial reddish-brown colour and become greyish-blue. Pure vitamin C (Fluka 95212) was used to prepare an ascorbic acid standard solution.

Fruit colour was measured at harvest and after three and four months of storage with a Minolta Chromameter (model CR-400) calibrated with a white standard tile. Only peel background colour was assessed and the results expressed as hue angle.

*Statistical methods*

To study the influence of 1-MCP on apple quality characteristics and loss of marketable yield, the data were analysed by analysis of variance (Figs. 1–4).

*Table 1*

Weather conditions in summer 2003 and 2004 in South Estonia: monthly mean air temperature (°C) and mean monthly precipitation (mm) as compared to averages of the same figures over many years (1966–1998) in Estonia

| Month | 2003 | | 2004 | | 1966–1998 | |
|---|---|---|---|---|---|---|
| | Temperature | Precipitation | Temperature | Precipitation | Temperature | Precipitation |
| May | 11.7 | 143 | 9.5 | 38 | 11.0 | 55 |
| June | 12.9 | 71 | 12.8 | 184 | 15.1 | 66 |
| July | 19.4 | 104 | 16.0 | 76 | 16.7 | 72 |
| August | 15.2 | 133 | 16.4 | 105 | 15.6 | 79 |
| September | 11.4 | 25 | 11.8 | 84 | 10.4 | 66 |

## Results

*Fruit dry matter, soluble solids, titratable acids and vitamin C*

The DM content of Talvenauding fruits was 13.2% and that of Krameri Tuviõun fruits 13.4% after harvest in the first experimental year. At the end of storage, the fruit DM content was 13.1 and 12.4%, respectively, and was not significantly influenced by 1-MCP treatment (data not shown). After harvest in the second experimental year, the fruit DM content was 11.5%. At the end of storage, the fruit DM content of control apples was 10.5% and that of treated apples 12.4% (data not shown). The influence of 1-MCP treatment on DM content was not significant.

The soluble solids content (SSC) of Talvenauding fruits was 11.3% and that of Krameri Tuviõun fruits 12.2% after harvest in the first experimental year. At the end of storage, the effect of 1-MCP treatment was significant in one cultivar, increasing the SSC of Talvenauding fruits (Fig. 1). After harvest in the second experimental year, the SSC of Talvenauding fruits was similar to that in the first experimental year: an average of 11.4%. At the end of storage, the SSC of control apples was significantly higher (11.4%) than that of treated apples (9.6%).

The content of titratable acids (TA) in Talvenauding fruits was 1.22% and in Krameri Tuviõun fruits 0.65% after harvest in the first experimental year. After four months of storage, the average fruit TA of Krameri Tuviõun was 0.33%, which was not influenced by 1-MCP treatment. The fruit TA of Talvenauding was 1.05 and 0.83% in the control and treated variant, respectively (Fig. 2). 1-MCP significantly reduced the fruit TA content in Talvenauding. After harvest in the second experimental year, the average TA content of Talvenauding fruits was 1.35%. After four months of storage, the fruit TA of Talvenauding was 0.52 and 0.69% in the control and treated variant, respectively. This time 1-MCP significantly increased the fruit TA content in Talvenauding.

The vitamin C content of Talvenauding fruits was 26 mg 100 g $FW^{-1}$ and that of Krameri Tuviõun fruits 19 mg 100 g $FW^{-1}$ after harvest in the first experimental year. After four months of storage, the average content of vitamin C in Krameri Tuviõun fruits was 25 and 23 mg 100 g $FW^{-1}$ in the control and treated variant, respectively, and was not significantly influenced by 1-MCP treatment (Fig. 3). The vitamin C content of Talvenauding fruits was 24 and 19 mg 100 g $FW^{-1}$ in the control and treated variant, respectively, and was significantly reduced by 1-MCP treatment. After harvest in the second experimental year, the average vitamin C content of Talvenauding fruits was 22 mg 100 g $FW^{-1}$. At the end of storage, the vitamin C content in the apples had dropped to 20 mg 100 g $FW^{-1}$ in 1-MCP-treated apples and to 18 mg 100 g $FW^{-1}$ in control apples. The effect of 1-MCP was significant, increasing the content of vitamin C.

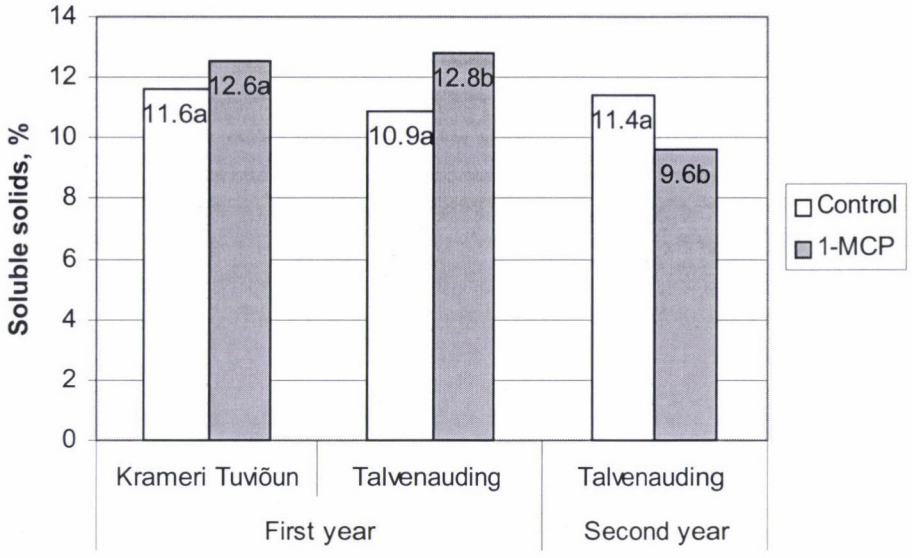



*Fig. 1.* Influence of 1-MCP treatment on the soluble solids content of Krameri Tuviõun and Talvenauding apples at the end of storage in two experimental years
Mean values followed by the same letter are not significantly different at $P \leq 0.05$

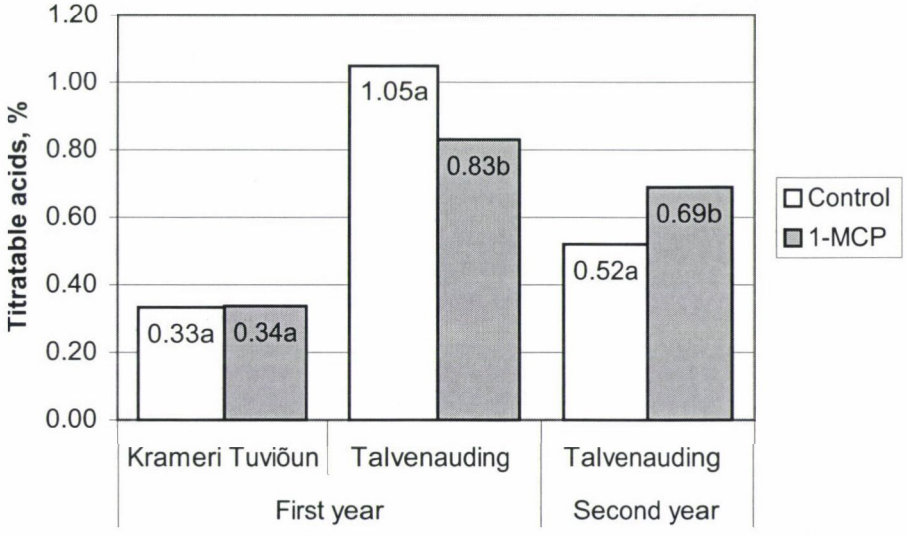



*Fig. 2.* Influence of 1-MCP treatment on the titratable acids content of Krameri Tuviõun and Talvenauding apples at the end of storage in two experimental years
Mean values followed by the same letter are not significantly different at $P \leq 0.05$

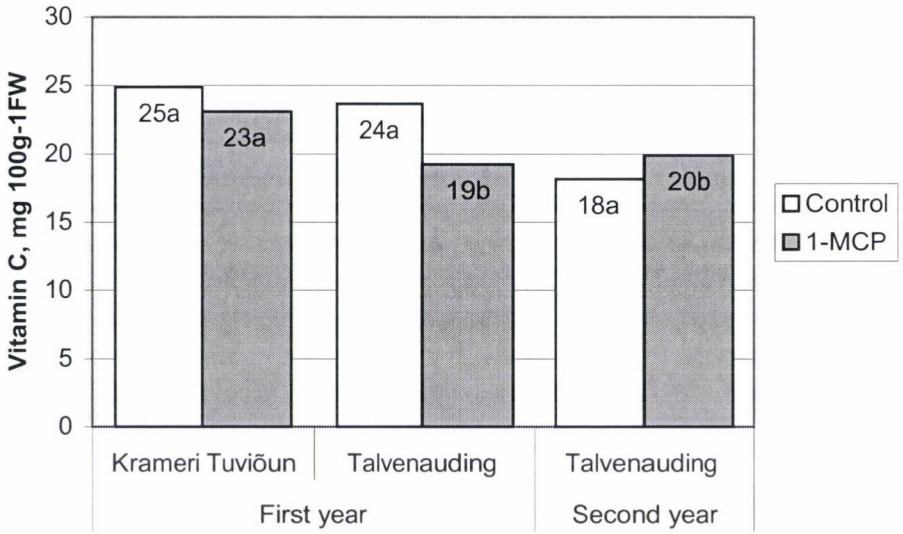



*Fig. 3.* Influence of 1-MCP treatment on the vitamin C content of Krameri Tuviõun and Talvenauding apples at the end of storage in two experimental years
Mean values followed by the same letter are not significantly different at $P \leq 0.05$

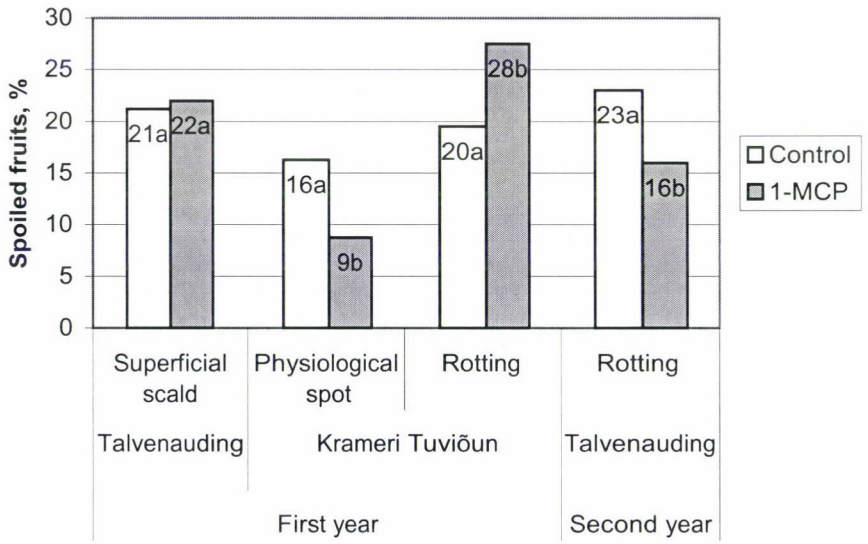



*Fig. 4.* Influence of 1-MCP treatment on superficial scald, rotting and physiological spot incidence in Krameri Tuviõun and Talvenauding apples at the end of storage in two experimental years
Mean values followed by the same letter are not significantly different at $P \leq 0.05$

*Physiological disorders and rotting*

In the first storage season the main reason for spoilage in Talvenauding was superficial scald, and 1-MCP treatment did not reduce this disorder (Fig. 4). In Krameri Tuviõun severe physiological spot and rotting caused notable damage, while bitter pit appeared in negligible amounts and is not further discussed. At the end of storage, 1-MCP treatment had a significant influence on both rotting and physiological spot in Krameri Tuviõun, but the influence was opposite: physiological spot in treated apples was reduced by almost 50%, while rotting increased by 40% (Fig. 4). In the second experimental year, the spoilage of Talvenauding was mostly due to rotting, which appeared after three months of storage. In this year no superficial scald was recorded. At the end of storage, the average amount of spoiled fruits was 23 and 16% in the control and treated variant, respectively, thus 1-MCP had reduced rotting by 30% (Fig. 4).

*Fruit firmness*

The fruit firmness of Talvenauding was 7.2 kg/cm$^2$ and that of Krameri Tuviõun 4.5 kg/cm$^2$ after harvest in the first experimental year. After four months of storage, the average fruit firmness of Krameri Tuviõun was 4.3 kg/ cm$^2$ in the control and 4.1 kg/cm$^2$ in the 1-MCP-treated variant. The fruit firmness of Talvenauding was 6.0 kg/cm$^2$ in both variants, so the effect of 1-MCP treatment was not significant (data not shown). In the second experimental year, the average fruit firmness of Talvenauding fruits was 5.2 kg/cm$^2$. At the end of storage, 1-MCP-treated fruits were significantly firmer, having values of 4.7 and 8.1 kg/cm$^2$ in the control and treated variant, respectively.

*Fruit colour*

During storage in the first experimental year, the hue angle (h) of the apple background peel colour in control fruit decreased from 109 to 95 in Talvenauding and from 106 to 96 in Krameri Tuviõun (Table 2). The results indicate a significant change from green to yellow during the storage of both cultivars. 1-MCP treatment had a significant influence on Talvenauding fruit peel colour after three and four months of storage: 1-MCP treated apples remained greener than control apples. The influence on Krameri Tuviõun was not significant. In the second experimental year 1-MCP treatment had no significant influence on Talvenauding fruit peel background colour; however, the tendency to increase the h value was still noted.

## Discussion

The results of the experiment confirmed that the influence of 1-MCP on apple cultivars differs. Similar observations were made previously by DeEll et al. (2002) and Watkins et al. (2000). Partial responses in some apple cultivars

*Table 2*
Talvenauding and Krameri Tuviõun apple peel background colour (hue angle) as affected by storage time and 1-MCP treatment in two experimental years

| | 2 months of storage | 3 months of storage | 4 months of storage |
|---|---|---|---|
| Talvenauding | | First year | |
| *Control* | 109.2aA | 97.8aB | 95.0aC |
| *1-MCP* | 109.3aA | 99.2bB | 96.5bC |
| Krameri Tuvion | | | |
| *Control* | 106.2bA | 97.4aB | 95.9cC |
| *1-MCP* | 106.4bA | 97.8aB | 96.0cC |
| | | Second year | |
| Talvenauding | | | |
| *Control* | 108.7aA | 101.2aB | 97.8aC |
| *1-MCP* | 108.7aA | 102.3aB | 98.8aC |

Small letters indicate significant differences ($P \leq 0.05$) due to 1-MCP treatment at each time of measurement. Capital letters indicate significant differences ($P \leq 0.05$) over time.

suggest that either these cultivars are able to regenerate new ethylene-binding sites, or that binding is incomplete. Apple cultivars produce different amounts of ethylene and some varieties might need higher 1-MCP concentrations to overcome the ethylene effect (Watkins et al., 2000). In the present experiment Krameri Tuviõun could be one of these cultivars, since it was considerably less influenced by 1-MCP treatment than the other cultivar. In order to explain the cause of cultivar differences, the ethylene production of the studied cultivars should be measured, and different concentrations of 1-MCP should be tested in the future.

The results of two years of experimentation on Talvenauding showed that the influence of 1-MCP might also be year-dependent. In the first year fruit firmness and superficial scald incidence were not influenced by 1-MCP treatment; the increase in SSC and the decrease in TA and vitamin C indicated that 1-MCP treatment might even speed up fruit ripening. Only the colour measurements did not confirm these findings, since treated apples remained greener. Regarding SSC, contradictory observations have been made by other scientists. Fan and Mattheis (1999) found that SSC was higher in 1-MCP-treated apples, while Rupasinghe et al. (2000) and DeEll et al. (2002) observed that soluble solids were reduced in 1-MCP-treated apples. 1-MCP treatment has been proved to delay the loss of titratable acidity (Pre-Aymard et al., 2003).

In the second year 1-MCP-treated fruits were firmer and contained more vitamin C, while SSC decreased and the loss of titratable acids was delayed. Consequently, fruit spoilage decreased and it can be concluded that in the second year, 1-MCP improved apple quality. Moran and McManus (2005) have reported that the effect of 1-MCP treatment on the coreline browning of Macoun apples was inconsistent, reducing its occurrence in 2002 and 2003, but increasing its occurrence in 2001, when the fruit were harvested at advanced maturity. Since the apples in the present experiments were harvested at normal

commercial maturity and the starch index was not determined, it is possible that the apples harvested in 2004, when the average air temperature was lower than in 2003, could have been less mature and the influence of 1-MCP therefore greater. At the same time, Watkins et al. (2000) found that the effectiveness of 1-MCP was not related to the harvest time of different cultivars.

The influence of 1-MCP on physiological disorders of apples has been intensely studied. Results have shown that 1-MCP substantially reduces the physiological disorders of apple fruit during storage, such as superficial scald (Rupasinghe et al., 2000; Watkins et al., 2000; Zanella, 2001; DeLong et al., 2004), soft scald (Fan et al., 1999), core browning, senescent breakdown (DeLong et al., 2004) and even bitter pit (Johnson, 2003). In the present experiment, 1-MCP reduced physiological spot incidence in Krameri Tuviõun by almost 50%, but did not influence superficial scald in Talvenauding. The development of scald is mostly related to the action of autooxidation products of α-farnesene in the apple skin, identified as conjugated trienes (Huelin and Coggiola, 1970). The synthesis of α-farnesene is influenced by ethylene (Watkins et al., 1993) and since 1-MCP blocks the response of plants to ethylene, it should also inhibit scald development. At the same time, it has been proposed that scald could result from more general oxidative stress, and the molecular genetic disruption of genes controlling α-farnesene biosynthesis is a strategy that should prove or disprove the direct role of α-farnesene oxidation in the induction of superficial scald.

The results suggest that the postharvest quality of Talvenauding fruits could be improved by 1-MCP treatment, but harvest maturity has to be taken into consideration. It was found that together with an improvement in storability and external quality characteristics, the biochemical content of Talvenauding fruits was also influenced. The most important aspect, from the consumer's point of view, was the influence of 1-MCP on the vitamin C content. Estonian apples are generally rich in vitamin C; according to long-term data from the Estonian Polli Horticultural Research Centre, the average vitamin C content of 18 Estonian apple cultivars is 15 m%, ranging from 6 to 30 m%. The vitamin C content of widely known commercial apple cultivars is usually lower: 12.8 mg 100 g $FW^{-1}$ was recorded in a study of six apple cultivars by Lee et al. (2003) and 5.7 mg by Eberhardt et al. (2000). It is important to find new postharvest technologies, which would improve storability and at the same time help to maintain the initial high content of the beneficial phytochemicals in apples. Therefore, further experiments with Nordic apple cultivars are needed to explain the reasons for the different influence of 1-MCP in different years.

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# PYROPHOSPHATE:FRUCTOSE 6-PHOSPHATE 1-PHOSPHOTRANSFERASE IS INVOLVED IN THE MOBILIZATION OF SUGAR RESERVES IN THE TAPROOTS OF COLD- AND DROUGHT-STRESSED CARROT PLANTS

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The purpose of this work was to further investigate the regulatory interplay between pyrophosphate:fructose 6-phosphate 1-phosphotransferase (PFP) and its positive effector, fructose 2,6-bisphosphate (Fru-2,6-$P_2$), in the storage organs of cold- and drought-stressed plants. Since there is no detectable cytoplasmic fructose-1,6-bisphosphatase (cytFBPase) activity in the taproots of carrot plants, PFP is the only enzyme that can replace its function when stored starch is converted to transportable sucrose. The working hypothesis was that PFP is likely to be involved in the mobilisation of energy reserves and might have a special role in storage organs such as carrot taproots upon stress. Both cold and drought stress resulted in a marked increase in the endogenous Fru-2,6-$P_2$ levels. It is suggested that the significant changes in photosynthate allocation are the direct results of the stimulation of PFP activity by elevated Fru-2,6-$P_2$ levels. PFP stimulated by Fru-2,6-$P_2$ operated in the gluconeogenic direction in the taproots of stressed carrot plants, whereas the glycolytic direction was dominant in the non-stressed controls. This suggests that the metabolic status determining the net activity of PFP depends on the physiological stress situation, making PFP an important sensor of environmental changes. The experimental data indicated that PFP is involved in the mobilisation of energy reserves during unfavourable environmental changes by promoting the re-synthesis of transportable sucrose in taproots.

**Key words:** PFP, fructose 2,6-bisphosphate, abiotic stress, glycolysis, gluconeogenesis, *Daucus carota*, taproot

**Abbreviations:** 6-PF-2-K/Fru 2,6-$P_2$ase = 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase gene; PFP = pyrophosphate:fructose 6-phosphate 1-phosphotransferase; PFK1 = 6-phosphofructo-1-kinase; Fru-2,6-$P_2$ = fructose 2,6-bisphosphate; 3PGA = 3-phosphoglycerate; hexose-P = hexose phosphate; FBPase = cytosolic fructose-1,6-bisphosphatase; Suc = sucrose; Glc = glucose; Glc-1-P = glucose 1-phosphate; Glc-6-P = glucose 6-phosphate; Fru-6-P = fructose 6-phosphate; PPi = pyrophosphate; WT = wild type

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## Introduction

Fru-2,6-$P_2$ is an important regulator of the photosynthetic carbon metabolism (for review see Stitt, 1990 and Nielsen et al., 2004). In leaves this signal metabolite contributes both to the coordination of sucrose synthesis with the rate of carbon dioxide fixation, and to the control of the partitioning of photosynthate between sucrose and starch (Scott et al., 1995; Truesdale et al., 1999; Toldi et al., 2002). The allosteric inhibition of cytosolic fructose-1,6-bisphosphatase (FBPase) by Fru-2,6-$P_2$ is central to the proposed mechanism by which this effector influences both of these processes (Stitt, 1997).

In contrast, the role of Fru-2,6-$P_2$ in non-photosynthetic plant tissues is poorly understood (Fernie et al., 2001). By analogy with animal and fungal systems it is frequently suggested that Fru-2,6-$P_2$ may contribute to the regulation of glycolytic flux (Stitt, 1990). The basis of this proposal is that non-gluconeogenic plant tissues often lack a detectable cytosolic FBPase activity (Entwistle and ap Rees, 1990). Therefore, any influence of Fru-2,6-$P_2$ on the metabolism must be attributed to the modulation of the glycolytic pathway, and thus respiration, through the activity of PFP in non-photosynthetic tissues of plants. The reaction catalysed by PFP in the cytosol of plant cells is close to equilibrium *in vivo* under normal physiological conditions (Weiner et al., 1987), meaning that PFP contributes equally to the gluconeogenic and glycolytic flux. However, when plants are subjected to environmental stressors this equilibrium may be modified.

It is known that Fru-2,6-$P_2$ provides adaptive abilities, by metabolic fine tuning, that are advantageous under suboptimal growth conditions (Okar et al., 2001; Nielsen et al., 2004). The Fru-2,6-$P_2$ signalling system responds sensitively to salt, drought, cold and osmotic stress by adjusting the fuel homeostasis according to the changing demand for survival (Reddy, 1996; 2000; Banzai et al., 2003; Villadsen et al., 2005). Storage organs like potato tubers and carrot taproots function as fuel reserves providing mobilisable energy sources under stress conditions. Fru-2,6-$P_2$ is involved in the regulation of the diurnal turnover of starch, which is the most important mobilisable energy source in higher plants, and has a pivotal role in stress adaptation. At the same time, PFP can substitute 6-phosphofructo-1-kinase (PFK) in maintaining glycolytic flux under ATP-limited stress situations by using PPi as phosphoryl donor. Since PFP is stimulated by Fru-2,6-$P_2$ allosterically, it is not an exaggeration to suppose that the functions of PFP and Fru-2,6-$P_2$ overlap in plant stress responses.

To validate this assumption, transgenic carrot plants with elevated levels of Fru-2,6-$P_2$ were produced and analysed. The taproots of these transgenic plants were used to investigate (i) whether PFP activity is altered as a result of elevated Fru-2,6-$P_2$ levels and (ii) whether there is any change in the direction of the net activity of PFP when taproots are subjected to different abiotic stressors (drought, cold).

## Materials and methods

*Plant material and growth conditions*

Carrot plantlets (*Daucus carota* L. cv. Nantes Duke) grown *in vitro* were obtained from Dr. S. Sorvari (MTT Agrifood Research, Piikkiö, Finland). After micropropagation and genetic transformation, transgenic and control plants were potted out and grown in peat-soil in a growth chamber with supplementary lighting of 12 h/150 mmol photons $m^{-2}$ $s^{-1}$ at 24°C and 70% relative humidity. Healthy, young taproots of 2-month-old plants were used for physiological measurements. Stressed lines were cultured in two ways prior to analysis. While half of the pots were grown at 10°C for 10 days, the other half of the pots were grown without further irrigation, also for 10 days. The plants were then re-watered and samples were taken for physiological measurements at noon, one day after re-watering.

*Plasmid constructs*

Functional 6-PF-2-K was provided by a modified coding region of the rat liver 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase gene (6-PF-2-K/Fru 2,6-$P_2$ase). The gene contained point mutations which changed serine-32 and histidine-258 to alanine (Tauler et al., 1991; Kurland et al., 1992). These modifications result in a functional enzyme which possesses no Fru 2,6-$P_2$ase activity, but can still make Fru-2,6-$P_2$ (Scott et al., 1995). This construct was identical to that described by Scott et al. (1995). A *Hind* III fragment containing 6-PF-2-K/Fru 2,6-$P_2$ase was inserted between a 35S CaMV promoter and a polyadenylation signal in the vector pJIT62. The final construct was digested with *Kpn* I and *Eco* RV, then filled in, and cloned into pBIN19 (Bevan, 1984). The pBIN19:6-PF-2-K construct was introduced into *Agrobacterium tumefaciens* strain LBA 4404, containing pAL4404, by direct transformation (Höfgen and Willmitzer, 1988).

*Plant transformation*

Somatic embryos from cell suspension cultures of carrot plants (*Daucus carota* L. cv. Nantes Duke) were infected with the transforming *Agrobacterium*. Co-culture took place by immersing embryo segments 2–3 mm long in 40 ml *Agrobacterium* suspension that contained bacterial cells at $OD_{660}$ = 0.5 density, one-third-strength MS macro- and microelements (Murashige and Skoog, 1962), half-strength MS vitamins, 1.0 g $l^{-1}$ casein hydrolysate, 100 μM acetosyringone and 10 g $l^{-1}$ glucose (pH 5.0) for 20–30 minutes at 22°C under dim light. After infection, the embryo segments were briefly dried and then placed on growth regulator-free MS medium solidified with 7 g $l^{-1}$ plant agar for the following 2–3 days. After the 2–3 day co-culture, plant explants were transferred into selective callus induction medium that contained 1.0 mg $l^{-1}$ 2,4-D, 100 mg $l^{-1}$ kanamycin and 500 mg $l^{-1}$ cefotaxime. The selection took 6–8 weeks and required subculturing every 2 weeks. The calluses were transferred into solid regeneration media that contained MS salts and vitamins, 1.0 mg $l^{-1}$ zeatin, 50 mg $l^{-1}$ kanamycin and 300 mg $l^{-1}$ cefotaxime. The plant regeneration required 16 h daylength conditions at 25°C. When shoots developed *de novo* reached 8–10 mm in length they were transferred onto solid MS media containing 1 mg $l^{-1}$ IBA for rooting. Viable plants with a well-developed root system were potted out in the greenhouse and cultivated for further examinations.

*Analysis of gene expression*

Total mRNA for RT-PCR analysis was obtained from extracts of wild type (WT) and transgenic carrot taproots by binding on a biotin-labelled oligodT probe fixed on streptavidin magnetic particles, which were then later isolated with a magnetic device. After mRNA elution with a low-ionic-strength solution, 2000 pg of the eluted mRNA was subjected to reverse transcription and DNA amplification in the presence of primers spanning a 620 bp long 6-PF-2-K/Fru-2,6-$P_2$ase fragment: 5'-GGGGCTCCTCCATACCA-3' and 3'-GCTCTGAACGTGGTCCTG-5'.

These primers were designed to be highly specific for mammalian 6-PF-2-K/Fru-2,6-$P_2$ase. Amplification has never been obtained when analysing total mRNA samples from WT plants. The constitutively expressed UBQ10 mRNA was used as an internal control for uniform loading, and amplified as a 220 bp UBQ10 fragment with ubiquitin primers: 5'-GGACCAGCAGCGTCTCATCTTCGCT-3' and 3'-CCGGAACATTATTAGGGACTACTTATTC-5'.

The RT-PCR amplification of the template was carried out using the following procedure: one cycle of 48°C for 45 min and 94°C for 2 min; 40 cycles of 94°C for 1 min, 58°C for 2 min and 68°C for 3 min; and finally one cycle of 68°C for 10 min. The amplified DNA was electrophoresed in 1.8% agarose and visualized by staining with ethidium bromide. The signals were quantified using Quantity One software (Bio-Rad) and normalized using ubiquitin expression. The linearity between mRNA concentration and the densitometric optical reading of the ethidium bromide reaction was verified by using increasing amounts of isolated total mRNAs.

*Extraction and measurement of enzyme activity*

Measurements were made on the non-photosynthetic taproots, using separate taproots from clonal carrot plants for each experimental sample.

Slices (ø = 10 mm × 2 mm thick) were cut from the centre of the taproot with a razor blade and immediately frozen in liquid nitrogen. Samples of about 500 mg $FW^{-1}$ were then homogenised to a fine powder at 4°C in a mortar and pestle in the presence of 5 ml of extraction medium containing 100 mM Hepes, 4 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA and 5 mM β-mercaptoethanol (Hajirezaei and Stitt, 1991). The homogenate was first centrifuged at 14.000 rpm for 5 min, and then the supernatant was assayed for 6-PF-2-K as described by Scott et al. (1995). These assay conditions were optimal for both mammalian and carrot enzyme. To check the reliability of the extraction and the measurement of 6-PF-2-K activity, recovery experiments were performed. Spinach leaf extracts, containing approximately the same activity of 6-PF-2-K as was expected to be present in the taproots, were added to the samples prior to homogenisation.

The PFP activities were first measured in the glycolytic and gluconeogenic directions separately, as described by Theodorou and Kruger (2001), and then total PFP activities were calculated by the addition of related data.

*Extraction and measurement of metabolites*

Cores of tissue (10 mm × 10 mm, approximately) were removed from the centre of the taproots, and slices (2 mm thick) were cut and instantly frozen in liquid nitrogen. Samples (1 g $FW^{-1}$) were homogenised in liquid nitrogen in a mortar and pestle. For all substrates except Fru 2,6-P, the homogenate was immediately suspended in 2 ml of 1.4 M perchloric acid and left on ice for 2 h. Then the extract was neutralised with 5 M $K_2CO_3$, and the insoluble debris removed by centrifugation twice at 10.000 g. The starch in the insoluble fraction was determined according to Morrell and ap Rees (1986). Sucrose and phosphorylated intermediates (3PGA, Glc-1-P, Glc-6-P, and Fru-6-P) were assayed in the soluble fraction by monitoring the enzyme-linked reduction of $NAD^+$ or the oxidation of NADH spectrophotometrically at 340 nm according to Scott and Kruger (1995). Fru-2,6-$P_2$ was extracted from the taproots and assayed as described by Scott and Kruger (1994). To confirm the reliability of the extraction and the measurement of the metabolic intermediates, recovery experiments were performed. This was done by adding to the sample, prior to extraction, an amount of the metabolite similar to that present in the taproots. The recovery of metabolites added to the carrot tissue was 90.7 ± 5.0% for Suc; 88.7 ± 6.6% for Glc; 89.8 + 9.4 % for Glc-6-P; 80.5 ± 4.5% for Glc-1-P; 69.2 ± 8.7 % for Fru-6-P; 89.1 ± 3.6 % for 3PGA and 83.2 ± 2.5% for Fru-2,6-$P_2$ (mean ± SE, where n = 3).

*Statistical analysis*

The significance of differences between treatment groups was determined using Student's *t*-test followed by Fisher's LSD test, as appropriate. If the differences were considered significant for $P < 0.05$, means were separated by LSD at $P = 0.05$. The results for continuous variables are expressed as means ± SD.

## Results

In the experiments, the regulatory interplay between PFP and its positive effector Fru-2,6-$P_2$ was investigated in carrot taproots, which possess a heterotrophic metabolism. The working hypothesis was that the integrated action of Fru-2,6-$P_2$ and PFP might have a role in mobilizing energy from storage organs such as carrot taproots when stressed. Plant responses to some abiotic stressors (drought, salt) involve a significant increase in endogenous Fru-2,6-$P_2$ levels. Besides analysing this group of plants, transgenic carrot plants with increased Fru-2,6-$P_2$ levels were produced in order to test and compare their physiological behaviour to WTs under stressed and ambient conditions. Carrot somatic embryo explants were transformed with T-DNA containing *npt* II as the selectable marker gene and a modified form of the 6-PF-2-K/Fru 2,6-$P_2$ase gene under the control of the CaMV 35S promoter. The kanamycin-resistant plants formed were then tested to confirm the expression of the 6-PF-2-K/Fru 2,6-$P_2$ase gene. The amounts of 6-PF-2-K/Fru-2,6-$P_2$ase transcript detected by RT-PCR showed a direct correlation to the activity of 6-PF-2-K (Fig. 1, Fig. 2A). Ubiquitin (UBQ) was amplified as an internal control to ensure uniform loading. The 6-PF-2-K activities covered a range of 140% to 490% of the WT values in transgenic plants during the light period (Fig. 2A), resulting in a proportional increase in Fru-2,6-$P_2$ contents (Fig. 2B). One line, T-PFK2-9, was chosen from this 'high expressing – high activity' group of plants for further examinations, while the 'low expressing – low activity' group was represented by line T-PFK2-2.

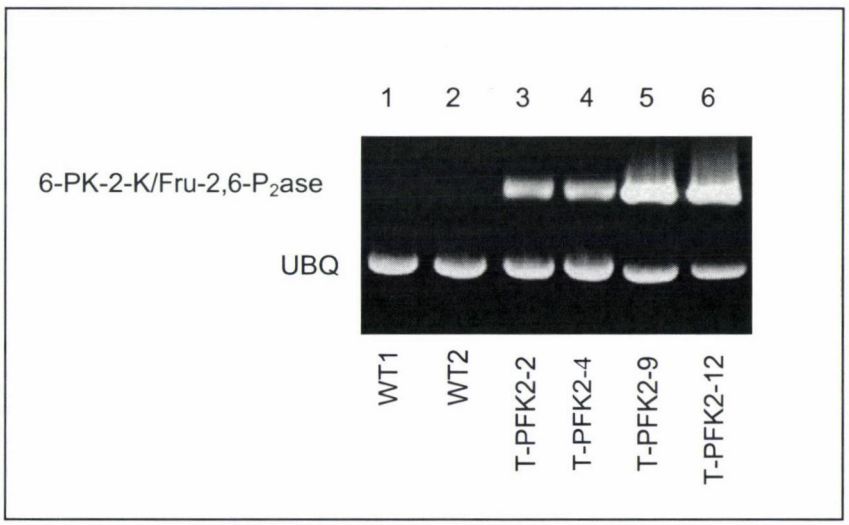



*Fig. 1.* Expression levels of 6-PF-2-K/Fru-2,6-P2ase analysed by RT-PCR in transgenic (3, 4, 5, 6) and WT plants (1, 2). Signals were quantified and normalized using ubiquitin (UBQ10) expression as a control. For mRNA extraction and measurements of 6-PF-2-K/Fru-2,6-P2ase activity, samples were harvested from taproots of 2-month-old plants at noon. All enzyme activity data are the mean ± SD of replicate measurements on three separate plants. Values labelled with asterisks are significantly different (Student's *t* test $P < 0.05$) from the corresponding data of non-stressed WT controls

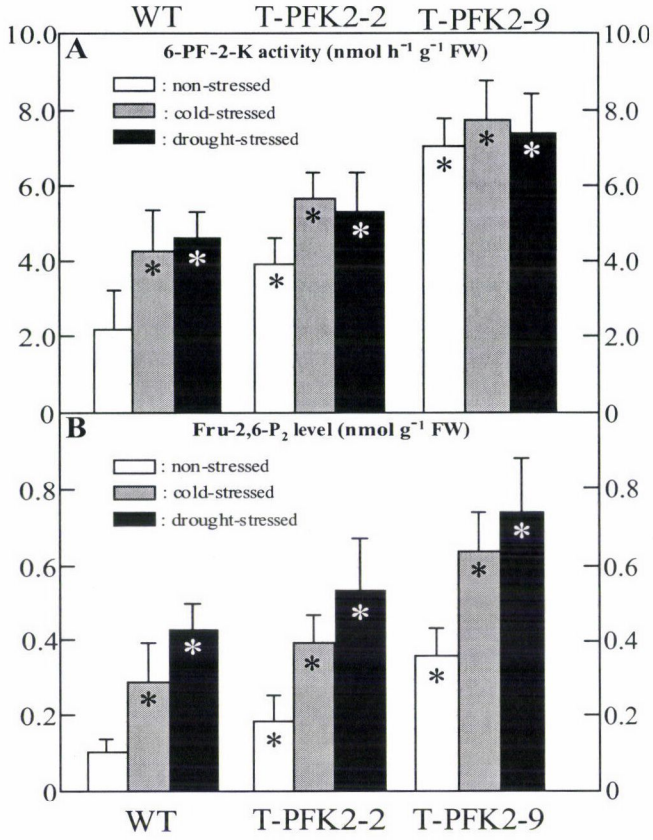



*Fig. 2.* Total activities of 6-phosphofructo-2-kinase (6-PF-2-K), responsible for synthetising Fru-2,6-$P_2$, and levels of Fru-2,6-$P_2$ in taproots of transgenic plant lines (T-PFK2-2 and T-PFK2-9) relative to WTs and the impact of cold and drought stress on these data. Samples were harvested from the taproots at noon. All data are the mean ± SD of replicate measurements on three separate taproots. Values labelled with asterisks are significantly different (Student's *t*-test $P < 0.05$) from the corresponding data of non-stressed WT controls

Exposure to cold and drought stress resulted in a significant increase in 6-PF-2-K activity and Fru-2,6-$P_2$ content both in transgenic (T-PFK2-2, T-PFK2-9) and control plants (Figs. 2A, 2B). However, this increase was more marked in the transgenic lines, especially in the high expressing T-PFK2-9 plants, where the Fru-2,6-$P_2$ levels were 200–300% of the corresponding values for WTs (Fig. 2B). There were no differences in the responses to cold or drought between the treatment groups.

Besides Fru-2,6-$P_2$, the concentrations of 3-phosphoglycerate (3PGA) and hexose phosphates (hexose-Ps) showed the most significant alterations (Fig. 3). While WT plants responded to cold and drought stress with rising 3PGA levels (175–220% of the non-stressed WTs), in transgenic lines the concentrations were just 10–25% of those in non-stressed counterparts by the end of the first

half of the light period (Fig. 3A). At the same time, changes in the concentration of hexose-Ps (glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate) showed the opposite tendency. While WT plants responded to cold and drought stress by reducing their hexose-P levels (10–25% of the non-stressed WTs), in the high expressing transgenic line (T-PFK2-9) the concentrations were 280–400% of the non-stressed counterparts by the end of the first half of the light period (Figs. 3B, 3C). This resulted in a marked shift in the 3PGA/hexose-P ratio in the transgenic lines relative to WTs. In WTs, the 3PGA/hexose-P ratios increased when the plants were subjected to cold or drought stress (Table 1). By contrast, cold and drought stress resulted in a strongly decreased 3PGA/hexose-P ratio in transgenic lines, which was paralleled by high ratios in the corresponding non-stressed controls. Transgenic taproots exhibited a marked increase in gluconeogenic PFP activity, providing an explanation for the above shift in the 3PGA/hexose-P ratio. This is one indication of a change in the direction of the PFP-catalysed reaction.

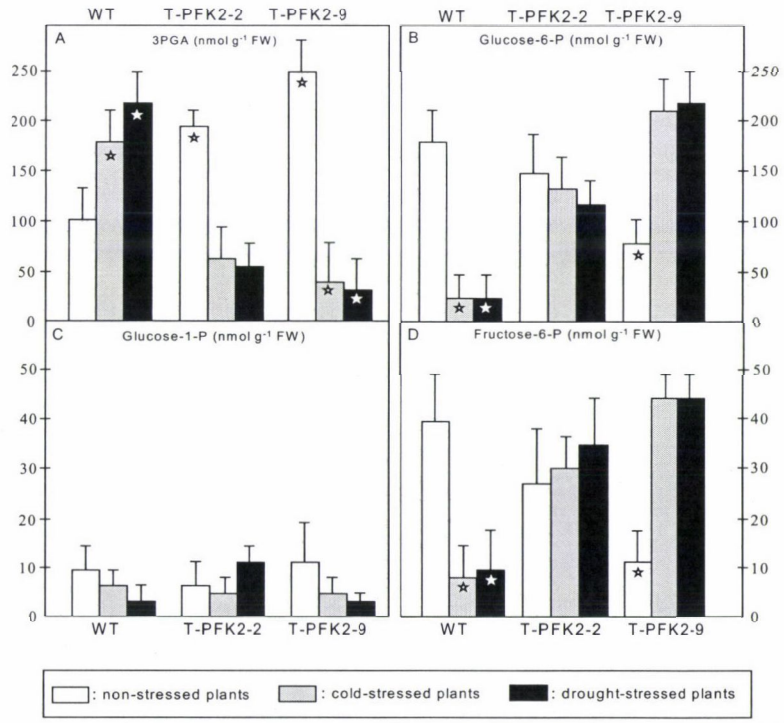



*Fig. 3.* Concentrations of phosphorylated intermediates (3PGA, Glc-6-P, Glc-1-P, Fru-6-P) in non-stressed (white bars), cold-stressed (grey bars) and drought-stressed (black bars) carrot taproots with elevated levels of Fru-2,6-$P_2$ (lines T-PFK2-2 and T-PFK2-9) compared to WTs. Samples were harvested from the taproots at noon. All data are the mean ± SD of replicate measurements on three separate taproots. Values labelled with asterisks are significantly different (Student's *t*-test $P < 0.05$) from the corresponding data of non-stressed WT controls

*Table 1*
Interrelation of the 3PGA/hexose-P ratio with the gluconeogenic and glycolytic activity of PFP in taproots with elevated levels of Fru-2,6-$P_2$ (transgenic plant lines T-PFK2-2 and T-PFK2-9) relative to WTs, and the impact of cold and drought stress on this interrelation

| Plant lines | Treatments | 3PGA/hexose-P ratio | Enzyme activity [μmol $min^{-1}$ $g^{-1}$ FW] | | |
|---|---|---|---|---|---|
| | | | PFP gluconeogenic | PFP glycolytic | PFP total |
| WT | Non-stressed | 1.00 | 0.09±0.02 | 0.11±0.02 | 0.20±0.04 |
| | Drought-stressed | 0.65 | 0.25±0.03 | 0.17±0.01 | 0.42±0.04 |
| | Cold-stressed | 0.70 | 0.26±0.04 | 0.14±0.02 | 0.40±0.06 |
| T-PFK2-2 | Non-stressed | 1.25 | 0.17±0.01 | 0.21±0.02 | 0.38±0.03 |
| | Drought-stressed | 0.43 | 0.38±0.04 | 0.13±0.01 | 0.52±0.05 |
| | Cold-stressed | 0.26 | 0.41±0.02 | 0.10±0.01 | 0.51±0.03 |
| T-PFK2-9 | Non-stressed | 1.29 | 0.19±0.03 | 0.24±0.02 | 0.43±0.05 |
| | Drought-stressed | 0.29 | 0.62±0.05 | 0.25±0.02 | 0.87±0.07 |
| | Cold-stressed | 0.31 | 0.67±0.04 | 0.15±0.02 | 0.82±0.06 |

The 3PGA/hexose-P ratio was considered to be 1.0 in the case of the non-stressed WT control. Samples were harvested from the taproots at noon. All data are the mean ± SD of replicate measurements on three separate taproots.

The second indication is the tendency of changes in the concentrations of major soluble sugars that possess osmotic capacity and provide directly metabolisable energy. Exposure to cold and drought stress resulted in a significant increase in the concentrations of sucrose, glucose and fructose both in transgenic (T-PFK2-2, T-PFK2-9) and control plants (Figs. 4A, 4C, 4D). However, this increase was more marked in the transgenic lines, especially in the high expressing T-PFK2-9 plants, where, for example, the sucrose levels were 175–190% of the corresponding values in WTs by the end of the first half of the light period (Fig. 4A). At the same time, changes in the concentration of insoluble starch showed the opposite tendency. While WT plants responded to cold and drought stress with a moderate decrease in starch levels (70–80% of the non-stressed WTs), in the high expressing transgenic line (T-PFK2-9) the concentrations were just 12–15% of the non-stressed counterparts by the end of the first half of the light period (Fig. 4B). There were no differences in the responses to cold or drought between the treatment groups. Since there was no detectable cytFBPase activity in the taproots (data not shown), the marked increase in sucrose in the transgenic lines can probably be attributed to the change in the direction of the PFP-catalysed reaction.

## Discussion

Phosphofructokinase (PFK1) and the cytosolic fructose-1,6-bisphosphates (cytFBPase) are thought to possess a determinative role in the control of glycolysis and gluconeogenesis in fungi and animals (Stitt, 1989). However, plants contain PFP, which is capable of substituting for both of these enzymes (ap Rees et al., 1985). Fru-2,6-$P_2$ activates PFP, but does not activate PFK1 in

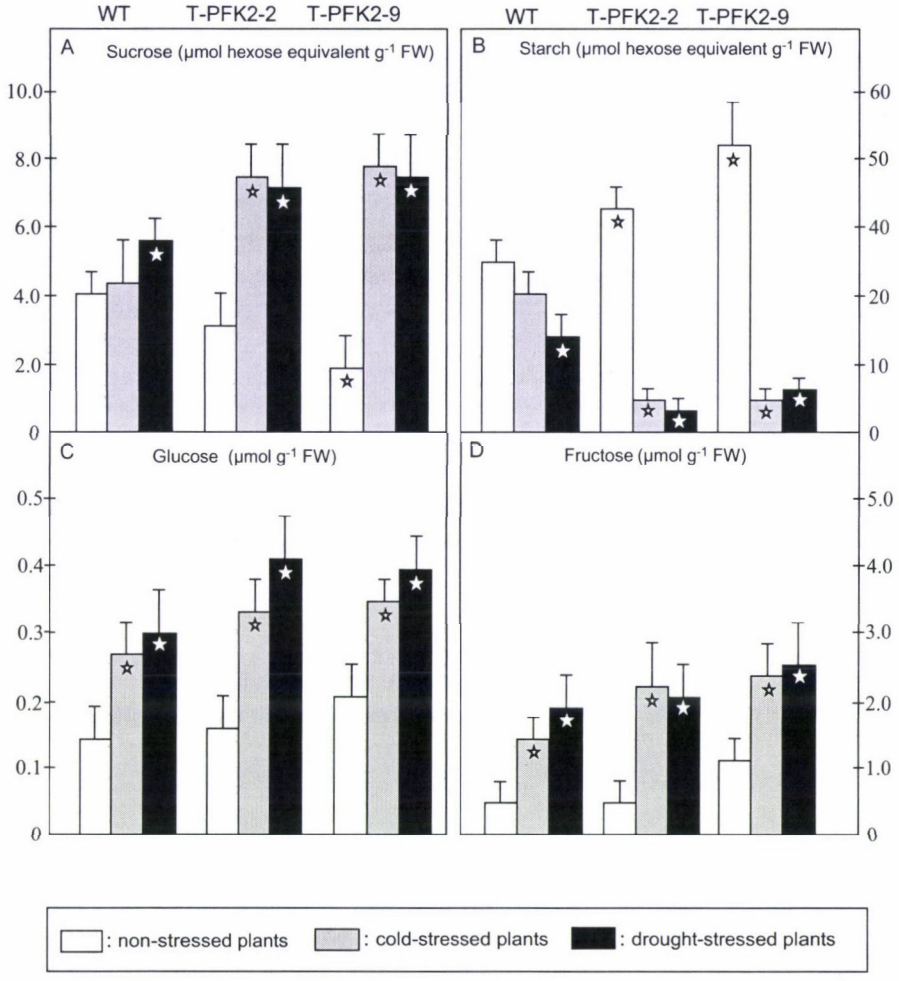



*Fig. 4.* Concentrations of soluble sugars (Suc, Glu, Fru) and starch in non-stressed (white bars), cold-stressed (grey bars) and drought-stressed (black bars) carrot taproots with elevated levels of Fru-2,6-$P_2$ (lines T-PFK2-2 and T-PFK2-9) compared to WTs. Samples were harvested from the taproots at noon. All data are the mean ± SD of replicate measurements on three separate taproots. Values labelled with asterisks are significantly different (Student's *t*-test $P < 0.05$) from the corresponding data of non-stressed WT controls

higher plants (van Schaftingen et al., 1982). Accordingly, it has been suggested that increasing concentrations of Fru-2,6-$P_2$ stimulate glycolysis in plants by activating PFP, in analogy to the action of Fru-2,6-$P_2$ on PFK1 in fungal and animal tissues (Cseke et al., 1987). However, measurements of metabolite levels have revealed that the reaction catalysed by PFP is close to equilibrium *in vivo*

(Edwards and ap Rees, 1986). In this case, PFP might equally well operate in the gluconeogenic direction. Indeed, several studies of Fru-2,6-$P_2$ levels and activities in contrasting tissues have been interpreted as evidence that the reverse reaction provides carbon for the synthesis of sugars, or PPi for sucrose mobilization via sucrose synthase (ap Rees et al., 1985; Edwards and ap Rees, 1986). Evidence has been provided that the endogenous levels of Fru-2,6-$P_2$ respond to salt, drought and osmotic stress with a prompt twofold increase (Banzai et al., 2003). The increasing Fru-2,6-$P_2$ levels certainly stimulate PFP activity, but the question is, in which direction? Since there is no detectable level of cytFBPase activity in carrot taproots and PFK1 operates in the glycolytic direction only, PFP is the only major enzyme that is regulated by Fru-2,6-$P_2$ and can replace the missing cytFBPase in the gluconeogenic route when the mobilization of starch reserves is required. To validate this assumption an experimental system was established, in which the endogenous Fru-2,6-$P_2$ level was challenged by cold and drought stress in carrot taproots, which store starch as a mobilisable energy reserve. Furthermore, the Fru-2,6-$P_2$ levels were elevated in carrot taproots by introducing a modified form of mammalian 6-PF-2-K/Fru-2,6-$P_2$ase into carrots, allowing us to analyse PFP in plants where the steady-state level of Fru-2,6-$P_2$ was higher even without stress.

The separated assay of the glycolytic and gluconeogenic activities of PFP provided experimental data proving that PFP operated in the glyconeogenic direction in the taproots of stressed carrot plants, whereas the glycolytic direction was dominant in the non-stressed controls (Table 1). Functional analysis of PFP has never been performed in other heterotrophic tissues subjected to different stressors. This explains why PFP has been generally considered as a glycolytic enzyme (Scott and Kruger, 1995; Fernie et al., 2001). The metabolic consequence of the net gluconeogenic activity of PFP was the rearrangement of carbohydrate partitioning (decrease in the 3PGA/hexose-P ratio and starch accumulation, paralleled by increased soluble sugar contents), which was considerably more marked in the transgenic plants than in the WTs (Fig. 3, Fig. 4). This suggests that the metabolic status determining the net activity of PFP depends on the physiological stress situation and, as such, PFP is an important sensor of environmental changes. Besides being a sensor enzyme, PFP also helps to mobilise energy reservoirs in response to unfavourable environmental changes by promoting the re-synthesis of transportable sucrose through gluconeogenesis from the starch accumulated in taproots.

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# EFFECT OF REDUCED TILLAGE ON QUALITY TRAITS OF SOYBEAN [*Glycine max* (L.) MERR.]

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Four different tillage systems were compared in soybean [*Glycine max* (L.) Merr.] production on one experimental field (chernozem) located in the Baranya region of northeastern Croatia in 2002 and 2003. The dry conditions experienced in 2003 exacerbated the negative effects of no-tillage on soybean yield. The 2-year average yield of soybean was significantly lower under no-tillage (NT) than in the conventional tillage (CT), soil loosening (SL) and disc harrowing (DH) treatments. The soybean oil and protein contents were very similar in all the tillage systems over the 2-year average. Soybean crude fibre (%) was affected by the main effect of tillage. Averaged over 2 years the crude fibre (%) of soybean grain was greater under NT than in the CT, DH and SL treatments. The ash (%) generally increased as tillage declined.

**Key words**: soybean [*Glycine max* (L.) Merr.], protein, oil, crude fibre, ash, yield, tillage

**Abbreviations**: CT: Conventional tillage; DH: Disc harrowing (fine till); SL: Soil loosening (chisel plough); NT: No-tillage; Y × TS: Year × tillage system; Ash: Mineral content.

## Introduction

Farmers have been encouraged to adopt no-tillage (NT) because of its environmental advantages compared with other conservation tillage systems (Yin and Al-Kaisi, 2004). Soybean [*Glycine max* (L.) Merr.] plants grown with no-tillage (NT) often appear smaller than those grown with conventional tillage (CT), yet they produce a similar grain yield (Yusuf et al., 1999).

No-tillage production results in changes in soil physical properties, including increases in soil organic matter content (Douglas and Goss, 1982), aggregate stability (Heard et al., 1988) and macroporosity (Lal et al., 1990). The changes may be detrimental, neutral or beneficial for crop growth and yield, depending on the soil texture and structure (Dick and VanDoren, 1985) and on

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climatic factors such as rainfall (Morrison et al., 2000). According to Wilhelm and Wortmann (2004) the soybean grain yield was less responsive to favourable environments after soil loosening with a chisel plough than in other tillage treatments.

In general, NT systems have a greater positive effect on crop growth and yield when used on soils characterized by low organic matter levels and poor structure, rather than on well-structured soils high in organic matter (Kladivko et al., 1986).

In a study by Košutić et al. (2001) the greatest soybean yield of 3460 kg $ha^{-1}$ was achieved in a conventional tillage system.

Soybean is known for its high protein and oil contents, with typical US cultivars averaging 410 and 210 g $kg^{-1}$ for protein and oil, respectively, on a dry weight basis (Leffel and Rhodes, 1993) However, Breene et al. (1988) reported a gradual decline in the protein content of soybean grown in the northern latitudes over a period of 10 to 15 years. Because of this decline, more emphasis has been placed on increasing the soybean protein content, but this goal has faced setbacks such as the negative relationship between seed and protein content (Escalante and Wilcox, 1993).

Nitrogen is considered to be a yield-determining factor in soybean production (Sinclair and De Wit, 1976; Frederick and Hesketh, 1994; Sinclair, 1998). Sinclair and De Wit (1976) postulated that the high demand for N in growing seeds of high-protein grain crops, such as soybean, cannot be satisfied by the daily N accumulation rates, so N must be remobilized from vegetative tissue. Most grain crops remobilize some vegetative N into the seed. In soybean, remobilized N has been estimated to contribute 20 to 60% of the N in the seed (Egli, 1988; Zeiher et al., 1982)

The aim was to assess tillage methods which are able to maintain high soybean yield and satisfactory grain quality properties, based on differences in oil content, protein content, crude fibre % and ash %.

## Materials and methods

Field experiments were conducted at Kneževo, in the Baranya region of northeastern Croatia (45°32' N and 18° 44' E, 90 m elevation). The study was conducted over a 2-year period (2002 and 2003) as a monofactorial trial with randomized plots divided into blocks within four replications, and with a basic plot area of 900 $m^2$ (18 × 50 m), on a chernozem soil, the dominant soil type of the Baranya region, with pH 8.06 ($pH_{(KCl)}$ 7.53), 2.61% organic matter, 187.0 mg $kg^{-1}$ P and 284.2 mg $kg^{-1}$ K (determined by the Egner-Riehm Domingo AL-method) and 2.12 % $CaCO_3$. The total precipitation (mm) and temperature (°C) from October to March (winter) and during the growing season (April to September) at the Kneževo site during 2002 and 2003 are shown in Table 1.

The experiment was conducted on the same homogeneous field at the same location in each experimental year. Prior to the start of the experiment only conventional tillage was applied. In both years the forecrop was winter wheat. From the eight tillage systems included in the long-term tillage experiment, four were selected for this study: conventional tillage (CT), disc harrowing (fine till) (DH), disc harrowing + soil loosening (SL) and no-tillage (NT).

*Table 1*
Total precipitation (mm) and temperature (°C) in winter (October to March) and during the growing season (April to September) at Kneževo in 2002 and 2003

| | 2002 (mm) | 2003 (mm) | 25-year mean | 2002 (°C) | 2003 (°C) | 25-year mean |
|---|---|---|---|---|---|---|
| Winter | 169 | 222 | 266 | 6 | 6 | 6 |
| April | 65 | 9 | 43 | 11 | 11 | 11 |
| May | 141 | 33 | 57 | 19 | 20 | 17 |
| June | 36 | 20 | 90 | 22 | 25 | 20 |
| July | 97 | 61 | 60 | 24 | 23 | 21 |
| August | 74 | 23 | 45 | 22 | 25 | 21 |
| September | 60 | 34 | 53 | 16 | 16 | 17 |
| Growing season | 473 | 10 | 348 | 19 | 20 | 18 |

The conventional tillage consisted of autumn ploughing (30 cm deep), the summer disking of wheat residues (7–10 cm deep), spring disc harrowing (15 cm deep), and disc harrowing to a depth of 10 cm and field cultivation in preparation for soybean. The disc harrowing (fine till) consisted of spring disc harrowing to a depth of 10–15 cm, followed by seeding. The disc harrowing + soil loosening (SL) consisted of autumn disc harrowing performed with a chisel plough to a depth of 25–30 cm, spring disc harrowing to a depth of 15 cm and seeding. The no-tillage treatment, by definition, involves no tillage or cultivation. A John Deere 750A grain drill was used for all tillage systems at a depth of 4 cm. The soybean cultivar Tisa was planted on 27 April 2002 and 4 April 2003 in 33-cm rows at a seeding rate of 100 kg $ha^{-1}$. In the year 2002 soybean emerged on May 15 and in 2003 on May 27. Fertilization was uniform for all tillage systems and both years (40 kg $ha^{-1}$ N, 130 kg $ha^{-1}$ P and 130 kg $ha^{-1}$ K as basic dressing).

Soybean grains harvested at maturity were sampled for the determination of oil and protein content. The grain yield was adjusted to 9% moisture. Several hundred seeds were selected randomly from the harvested grain of each plot and dried in a forced-air oven at 60°C for 24 h. A 12-seed subsample from each experimental unit was then ground to pass through a 1-mm screen. The total N of the seed was determined using a micro-Kjeldahl digestion procedure (Nelson and Sommers, 1980) and grain protein was estimated as 6.25 × N. The oil content of the grain from each tillage system was determined using a Soxhlet extraction method (AOAC, 1985).

The influence of different tillage systems on the quality traits of soybean was investigated by variance analysis and tested using the F-test ($P<0.01$**; $P<0.05$*).

## Results and discussion

The long-term monthly precipitation and air temperature means at Kneževo, in the Baranya region, and the average total precipitation and temperature during the growing season for 2002 and 2003 are presented in Table 1. In general, 2002 was wetter and cooler than the long-term mean. In contrast, with the exception of April, 2003 was drier than the mean during the growing season and slightly warmer than the mean, particularly in May and September (Table 1).

The increased drought stress in 2003 was probably responsible for the lower seed yields observed in 2003 (Table 2), by exacerbating the negative effects of NT on soybean yield. Analysis of variance indicated that the soybean yield was affected by the main effects of both the year and the tillage system (Table 2). Grain yields were similar for the CT and NT treatments in the study of

Yusuf et al. (1999). In the present study the yield of soybean was significantly lower under NT than in the CT, SL and DH treatments in both years (Table 2). No-tillage is detrimental to early-season plant growth, but does not usually substantially decrease the grain yield of soybean (Kladivko et al., 1986).

The year × tillage system interaction was significant and could also possibly explain why higher soybean yields were recorded in 2002 in all the tillage systems than in 2003 in the present study (Table 2). The results were in accordance with those of many authors who emphasized the importance of the climate conditions during the growing season in the development of the grain yield (Morrison et al., 2000; Košutić et al., 2001; Wilhelm and Wortmann 2004).

The soybean protein and oil contents were similar in all the tillage systems over a 2-year average, which is in accordance with previous experiments (Yusuf et al., 1999). The soybean protein content was significantly affected by the main effects of both year and tillage, but the oil content was only affected by the main effect of tillage (Table 2).

Soybean grain contained more protein in 2002 than in 2003 (32.27 g $kg^{-1}$; 30.36 g $kg^{-1}$), but the oil content was very similar in both years (20.40 g $kg^{-1}$ ; 20.06 g $kg^{-1}$). The inverse relationship between grain oil and grain protein is well known (Scott and Aldrich, 1983). Differences in the grain protein and oil responses between the years were probably due to the temperature. Cool conditions during grain fill generally result in a decrease in grain oil and an increase in grain protein (Calvin, 1965). In the present study the air temperature during the grain-filling period (mid- to late August and early September) and during the wet period in September was cooler in 2002 than in 2003 (Table 1).

Selection for protein and oil content in short, single-row trials at one location appears to be just as effective as selection in long, multiple-row trials at multiple locations, although year effects may have an impact (Pazdernik et al., 1996).

*Table 2*
Analysis of variance and mean for quality traits of soybean as affected by reduced tillage at Kneževo in 2002 and 2003

| | Grain protein (%) | Grain oil (%) | Crude fibre (%) | Ash (%) | Grain yield kg $ha^{-1}$ |
|---|---|---|---|---|---|
| 2002 | 32.27 | 20.48 | 5.73 | 4.97 | 3174 |
| 2003 | 30.36 | 20.06 | 5.71 | 5.05 | 1964 |
| CT | 31.34 | 20.23 | 5.33 | 4.89 | 2834 |
| DH | 31.09 | 19.75 | 5.74 | 4.95 | 2635 |
| SL | 31.06 | 20.42 | 5.68 | 5.14 | 2730 |
| NT | 31.17 | 20.51 | 6.13 | 5.07 | 2076 |
| | | | F-values | | |
| Year | 549.037** | $1.925^{NS}$ | $0.043^{NS}$ | $4.703^{NS}$ | 14.311** |
| Tillage | 3.623* | 7.362** | 3.168* | 5.440** | 18.219** |
| Y ×TS | $0.355^{NS}$ | $2.233^{NS}$ | $3.638^{NS}$ | $0.977^{NS}$ | 4.272* |

CT: Conventional tillage; DH: Disc harrowing (fine till); SL: Soil loosening (chisel plough); NT: No-tillage; ** Significant at the 0.01 level; * Significant at the 0.05 level; $^{NS}$ Non-significant

The crude fibre (%) was affected by the main effect of tillage. Averaged over two years the crude fibre (%) of soybean grain was greater under NT than in the CT, DH and SL treatments (Table 2).

Analysis of variance indicated that the soybean ash % was affected by the main effect of tillage. The ash % was greater in SL and NT than in the CT and DH treatments, averaged over two years. The ash % generally increased as tillage declined. The increase in the grain ash % indicates that the stems of soybean plants growing under SL and NT conditions were thicker than those of soybean plants growing under CT and DH conditions for most of the season. Environmental stress during reproductive growth may alter the mineral composition of soybean seeds, affecting their nutritional value, functional quality and seed quality. The changes in mineral concentration resulting from high temperature may alter the nutritional quality of soybean and modify its end-use properties for oil processing and tofu production (Gibson and Mullen, 2001).

No year × tillage system interaction was observed for soybean protein and oil content, crude fibre % or ash % (Table 2). This indicates that the ranking of the soybean cultivars for protein and oil content, crude fibre and ash was unaffected by the tillage system (Table 2). The response of soybean quality traits to the tillage system varied with the prevailing weather conditions in the particular growing season.

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# COMPARISON OF DIFFERENT METHODS USED IN CALCULATING THE EFFECT OF STRIPE RUST ON WHEAT GRAIN YIELDS

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One of the most important diseases of wheat in Turkey is yellow rust. The severe epidemic in the 1997–1998 growing season, which caused significant yield reductions, and the absence of infection at the Hamidiye Substation of the Anatolian Agricultural Research Institute made it possible to calculate the yield losses due to stripe rust. This calculation was based on yield differences between genotypes at infected and disease-free locations using various methods.

Using the method suggested by Campbell et al. (1975) the calculated yield loss due to stripe rust varied among genotypes and locations with an overall range of 12.7 to 87.0%.

By the second method, entries were divided into five groups according to their Average Coefficients of Infection (ACI) and the yield losses in each group were calculated. Yield reductions when ACI was over 70 reached up to 57.5 % in Regional Yield Trials.

In the third method, regression analysis was applied to estimate the effect of ACI on grain yields. A highly significant linear relationship was found between the ACI values of the entries and their grain yields, with an estimate of 21.4 kg/ha yield reduction per unit increase in ACI.

**Key words:** wheat, stripe rust, yield loss evaluation

## Introduction

Wheat stripe rust is one of the most important wheat diseases in Turkey, as in most parts of the world. The widespread presence of wild relatives of wheat, due to the country's being one of the main homes of wheat in the Fertile Crescent, and the high altitude, causing cool, rainy springs, make stripe rust a significant disease. In Turkey, which has more than 9 million hectares of wheat, stripe rust is very common in high, cool regions like Central and Eastern Anatolia and Thrace, and in mountainous areas (Iren, 1981). In the last 25 years, stripe rust has caused severe epidemics once every 3–5 years. It caused damage in 1975, 1976, 1977, 1981 and 1991, either in the whole country or in some

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regions (Braun and Saari, 1992). Stripe rust is generally accepted as a low temperature pathogen, being most virulent in cool, rainy weather (Stubbs, 1988). *P. striiformis* has the lowest temperature requirements among the three wheat rust pathogens. Minimum, optimum and maximum temperatures for stripe rust infection are 0, 11 and 23°C, respectively. *P. striiformis* can actively overwinter on autumn-sown wheat.

The potential to cause epidemics and the ability to spread over long distances are some other reasons why the disease is important. In addition, stripe rust is more detrimental to grain yield than stem rust or leaf rust, due to its earlier occurrence during the growing period (Stubbs, 1985).

The size of the epidemic depends on the virulence of the pathogen, the susceptibility of the cultivars grown and the suitability of the environmental conditions. Cool, rainy springs and a high acreage of the highly susceptible variety Gerek79, particularly in the major wheat areas of the Central Plateau, have resulted in significant production losses in recent years (Bolat et al., 1999; Yildirim et al., 1999). Roelfs et al. (1992) gave valuable information on the methods used to calculate yield losses due to rusts. Doling and Doodson (1968) and Mundy (1973) summarized the equations they developed to calculate yield losses due to stripe rust.

In a study carried out in Tel-Hadia, Syria, yield losses caused by stripe rust were 12% and 7% in the 1997–1998 growing year, with and without supplemental irrigation, respectively, and 16% and 5% in the following year (Ketata, 2001). According to the same researcher, the yield losses varied among varieties from 0 to 50% in the 1995–1996 season. In another study, yield losses due to stripe rust were found to be 51 and 46% for irrigated and rainfed areas, respectively (Smith et al., 1986). In Chile, yield losses due to rusts, calculated using 30 years of data, were reported to be 6.25% of the total harvest (Hacke, 1992). Stripe rust was reported to cause up to 84% yield loss in New South Wales, Australia, between 1984 and 1987 (Murray et al., 1994). According to the same researchers, stripe rust causes the greatest yield loss during the early milky dough stage, and the rate of this loss increases with the duration of the epidemic. Another study also revealed the importance of the time of occurrence of the epidemic, showing that early occurring epidemics caused 30 to 96% yield loss, while even severe epidemics did not reduce yields more than 10–15% when they occurred in later growth stages (Kaidash et al., 1976).

In a study using genotypes with or without the Yr 18 gene, which enables slow rusting, this gene provided 36 to 58% yield protection, depending on years and locations (Ma and Singh, 1996). In another study, comparing genotypes with adult stage resistance with a susceptible genotype, yield losses due to stripe rust were 15–25% and 45–50%, respectively (Park et al., 1988). In research carried out in an area of intensive disease, using $F_1$ and $F_2$ generations out of crosses among 8 bread wheat genotypes, the total yield loss caused by diseases was 33%, 25% of which was due to stripe rust (Wagoire et al., 1998).

The yield component most affected by stripe rust is kernel weight. The 1994–1995 epidemic in Cukurova, Turkey, caused 8.6 to 57.4% reduction in the

kernel weight of the susceptible variety Seri82 (Dusunceli et al., 1996). Braun and Saari (1992) calculated yield losses due to stripe rust by comparing the yields of Gerek79 with that of four other standards in the regional yield trial in Eskisehir, Turkey, and reported a loss of 26.5% for the susceptible Gerek79 in an epidemic year.

In the spring of 1998, severe stripe rust epidemics occurred in the Central Plateau and the Transitional Regions of Turkey, causing significant yield losses in widely grown cultivars, particularly in Gerek79. In four of the five locations where the Anatolian Agricultural Research Institute conducted regional yield trials, moderate or severe epidemics occurred, while at the Hamidiye Substation stripe rust was not observed (Bolat et al., 1999). This difference made it possible to calculate yield losses due to stripe rust.

The objective of this work was to study the effect of stripe rust on the grain yield of wheat and to compare the different methods used to calculate yield losses.

## Materials and methods

The climatic conditions of the 1997–1998 wheat growing season resulted in severe stripe rust epidemics in all locations of the regional yield trials except for one. The grain yields of standard varieties in locations with epidemics were compared with those in the Hamidiye location where rusts were not observed. Stripe rust readings on the lines in the experiment were taken using the Modified Cobb Scale. Average Coefficients of Infection (ACI) were calculated as suggested by Stubbs et al. (1986). Four susceptible (Gerek79, Kirgiz95, Gun 91 and Kutluk94) and one resistant (Sultan95) standard varieties, and eight and three advanced lines from the 1st RYT (Regional Yield Trial) and the 2nd RYT, respectively, were included in five locations.

The experimental layout was a randomized complete block design with four replications for all trials. Plot dimensions were $0.2 \times 6 \times 6 = 7.2$ m$^2$ at planting and $0.2 \times 6 \times 5 = 6$ m$^2$ at harvest.

Data from a preliminary yield trial (PYT) with two replications and a yield trial (YT) with three replications were used for this purpose. In PYT and YT, the plot sizes were $0.2 \times 4 \times 5 = 4$ m$^2$ and $0.2 \times 6 \times 5 = 6$ m$^2$ at harvest, and the number of lines were 517 and 121, respectively.

Three different methods were used to calculate yield losses. The first method used, suggested by Campbell et al. (1975), is shown by Equation 1.

$$Lx = (Xn - Xi) - (\bar{R}n - \bar{R}i) \qquad \text{Equation 1}$$

where Lx = yield loss of susceptible genotypes in locations with epidemics; Xn = yield of a susceptible genotype in the location without rust; Xi = yield of a susceptible genotype in locations with epidemics; $\bar{R}n$ = average yield of resistant genotypes in the location without rust; $\bar{R}i$ = average yield of resistant genotypes in the locations with epidemics.

This equation was applied to calculate the yield losses of the susceptible standards due to stripe rust, using the average yields of the resistant standard and lines in locations with and without rust, in order to eliminate the effect of other yield-affecting factors.

In the second method, the varieties and lines involved in the experiment were divided into five groups based on their ACI. The yields in the first group of lines (with ACI of 0 to 10) were assumed to be unaffected by stripe rust. The yield losses of the susceptible lines were calculated using Equation 2, as suggested by Bockus and Niblet (1984):

$$Ls = (\bar{S}b - \bar{S}a) - (\bar{R}b - \bar{R}a) \qquad \text{Equation 2}$$

where Ls = average yield loss of the susceptible group of lines in locations with epidemics; $\bar{S}b$ = average yield of the susceptible group of lines in locations with epidemics; $\bar{S}a$ = average yield of the susceptible group of lines in the location without rust; $\bar{R}b$ = average yield of resistant lines in locations with epidemics; $\bar{R}a$ = average yield of resistant lines in the location without rust.

The third method was to study the relationship between the grain yields of the lines in PYT, YT and RYT and their coefficient of infection through regression analysis. This method was applied at the Eskisehir location on 697 entries.

## Results

The data used to calculate yield losses due to stripe rust were obtained from four susceptible and one resistant standards and eleven advanced lines, planted in a total of ten trials set in five locations. The locations of the trials, varieties and lines in the experiment, and their stripe rust readings are presented in Table 1. There was no epidemic in Hamidiye and this provided an opportunity to calculate yield losses due to the disease. The most severe epidemics occurred in the Eskisehir and Altintas locations, followed by Banaz and Sivasli. In all locations, the greatest effect of stripe rust was observed on the variety Gerek79, followed by Kirgiz95, Gun91 and Kutluk94. The stripe rust readings of the resistant variety, Sultan95, and the promising lines in the trials were either zero or very close to zero.

In the 1st and 2nd RYT, while the difference between the average yields of susceptible and resistant varieties and lines was not significant in Hamidiye, where stripe rust was not observed, the average yields of susceptible varieties were significantly lower than those of resistant varieties and lines in other locations, where stripe rust epidemics occurred (Table 2). The greatest yield differences occurred in the Altintas location, followed by Eskisehir and Banaz, while the lowest yield difference was recorded in Sivasli.

*Table 1*

Names, origins and stripe rust reactions of the wheat varieties and lines used in the experiments

| Variety/Line | Origin | Reactions at the locations* | | | | |
|---|---|---|---|---|---|---|
| | | Eskisehir | Altintas | Banaz | Sivasli | Hamidiye |
| Gerek79 | Turkey (Cultivar) | 100 | 100 | 97 | 70 | 0 |
| Kirgiz95 | Turkey (Cultivar) | 100 | 70 | 62 | 53 | 0 |
| Gun91 | Turkey (Cultivar) | 62 | 52 | 45 | 33 | 0 |
| Kutluk94 | Turkey (Cultivar) | 53 | 48 | 44 | 30 | 0 |
| Sultan95 | Turkey (Cultivar) | 0 | 0 | 0 | 0 | 0 |
| 1st RYT-6 | Turkey (Line) | 2 | 0 | 3 | 0 | 0 |
| 1st RYT-13 | Turkey (Line) | 10 | 0 | 0 | 0 | 0 |
| 1st RYT-16 | Turkey (Line) | 3 | 0 | 2 | 0 | 0 |
| 1st RYT-17 | Turkey (Line) | 0 | 0 | 0 | 0 | 0 |
| 1st RYT-18 | Turkey (Line) | 1 | 10 | 2 | 0 | 0 |
| 1st RYT-22 | Turkey (Line) | 2 | 6 | 2 | 6 | 0 |
| 1st RYT-23 | Turkey (Line) | 1 | 0 | 0 | 0 | 0 |
| 1st RYT-24 | Turkey (Line) | 0 | 0 | 0 | 0 | 0 |
| 2nd RYT-9 | Turkey (Line) | 1 | 10 | 0 | 0 | 0 |
| 2nd RYT-14 | Turkey (Line) | 4 | 0 | 0 | 0 | 0 |
| 2nd RYT-15 | Turkey (Line) | 15 | 2 | 1 | 0 | 0 |

*Average Coefficient of Infection

*Table 2*
Yield performances of susceptible and resistant varieties in locations with and without stripe rust epidemics

| Experiment No. | Location | Average yield | | Ratio (100×S/R) | Significance of susceptible vs resistant varieties |
|---|---|---|---|---|---|
| | | Resistant varieties (R) (t/ha) | Susceptible varieties (S) (t/ha) | | |
| 1st RYT | Hamidiye + | 3.37 | 3.76 | 111.6 | NS |
| | Eskisehir ++ | 4.65 | 2.66 | 57.2 | ** |
| | Sivasli++ | 3.87 | 3.37 | 87.1 | ** |
| | Banaz++ | 4.25 | 2.45 | 57.6 | ** |
| | Altintas++ | 4.74 | 1.87 | 39.5 | ** |
| 2nd RYT | Hamidiye | 4.36 | 4.25 | 97.5 | NS |
| | Eskisehir | 4.95 | 2.74 | 55.3 | ** |
| | Sivasli | 3.68 | 2.88 | 78.3 | ** |
| | Banaz | 3.71 | 2.29 | 61.7 | ** |
| | Altintas | 4.60 | 2.10 | 45.7 | ** |

+=without epidemics; ++=with epidemics; **=Significant at P<0.01; NS=not significant

The yield losses calculated for the varieties in different locations using the first method are summarized in Table 3. The greatest yield loss was suffered by the variety Gerek79 with 87.0% and 83.6% in the 1st and 2nd RYT, respectively, in Altintas, followed by 68.8% and 64.7% yield loss for the same variety in the 1st RYT in Eskisehir and Banaz, respectively. This variety was followed by Kirgiz95, Gun91 and Kutluk94 in this respect. When the locations were compared for the yield losses of the varieties, Altintas had the greatest yield loss, followed by Eskisehir and Banaz, while the smallest yield reduction occurred in Sivasli.

In the evaluation based on the second method, a total of 697 varieties and lines were studied in PYT, YT and RYTs set up in the Eskisehir and Hamidiye locations, the varieties and lines being divided into five groups according to their ACI (Tables 4, 5 and 6). In addition to Eskisehir and Hamidiye, the Altintas, Banaz and Sivasli locations were used for RYT. Reductions in grain yield increased with the increasing severity and intensity of stripe rust. According to the results of PYT and YT in Eskisehir and RYT in various locations, when the stripe rust CI was more than 70, the yield losses in PYT, YT and RYT were 41.7, 39.2 and 57.5%, respectively (Tables 4, 5 and 6).

*Table 3*
Yield losses (%) in susceptible wheat varieties at different locations

| Location | Experiment | Gerek79 | Kirgiz95 | Gun91 | Kutluk94 | Average |
|---|---|---|---|---|---|---|
| Eskisehir | 1st RYT | 56.9 | 55.8 | 35.7 | 41.2 | 47.40 |
| | 2nd RYT | 68.8 | 47.2 | 30.8 | 32.7 | 44.88 |
| Sivasli | 1st RYT | 29.7 | 17.2 | 23.7 | 14.4 | 21.25 |
| | 2nd RYT | 12.7 | 25.1 | 22.9 | 14.2 | 18.73 |
| Banaz | 1st RYT | 64.7 | 48.6 | 44.3 | 33.5 | 47.78 |
| | 2nd RYT | 40.3 | 44.3 | 52.7 | 36.9 | 43.55 |
| Altintas | 1st RYT | 87.0 | 72.2 | 47.4 | 50.4 | 64.25 |
| | 2nd RYT | 83.6 | 58.7 | 41.0 | 37.2 | 55.13 |
| Average | | 55.46 | 46.14 | 37.31 | 32.56 | 42.87 |

*Table 4*
Relationship between ACI and yield losses (%) in PYT

| Coefficient of infection | Number of entries | Yield (t/ha) | | Yield loss (%) |
|---|---|---|---|---|
| | | Eskisehir | Hamidiye | |
| 0.0–10.0 | 183 | 4.93 | 2.89 | 0.0 |
| 10.1–30.0 | 121 | 4.43 | 2.73 | 7.1 |
| 30.1–50.0 | 122 | 4.04 | 2.71 | 15.0 |
| 50.1–70.0 | 63 | 3.58 | 2.58 | 22.5 |
| 70.1–100.0 | 28 | 2.88 | 2.90 | 41.7 |
| Total | 517 | | | |

*Table 5*
Relationship between ACI and yield losses (%) in YT

| Coefficient of infection | Number of entries | Yield (t/ha) | | Yield loss (%) |
|---|---|---|---|---|
| | | Eskisehir | Hamidiye | |
| 0.0–10.0 | 28 | 4.91 | 2.91 | 0.0 |
| 10.1–30.0 | 32 | 4.90 | 3.15 | 4.9 |
| 30.1–50.0 | 36 | 4.55 | 3.01 | 9.2 |
| 50.1–70.0 | 22 | 4.15 | 3.09 | 18.5 |
| 70.1–100.0 | 3 | 3.12 | 3.13 | 39.2 |
| Total | 121 | | | |

*Table 6*
Relationship between ACI and yield losses (%) in RYT*

| Aver. coeff. of infection | Yield (t/ha) | | | | Yield loss | | | | Average |
|---|---|---|---|---|---|---|---|---|---|
| | Eskisehir | Altintas | Banaz | Sivasli | Eskisehir | Altintas | Banaz | Sivasli | |
| 0.0 – 10.0 | 4.80 | 4.65 | 3.74 | 3.68 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 10.1 – 30.0 | 4.54 | 3.79 | 3.33 | 3.56 | 2.1 | 19.4 | 11.1 | 7.1 | 9.9 |
| 30.1 – 50.0 | 3.80 | 2.80 | 2.90 | 3.25 | 20.4 | 41.2 | 22.5 | 15.9 | 25.0 |
| 50.1 – 70.0 | 3.40 | 2.30 | 2.55 | 2.93 | 27.9 | 53.4 | 41.1 | 23.4 | 36.5 |
| 70.1 – 100.0 | 1.99 | | 1.42 | | 57.5 | | 55.0 | | 56.4 |

* Total number of entries was 59

Similarly, the regression analysis between grain yields and coefficient of infection for the lines in the Eskisehir trials showed that the yields significantly decreased as the infection constants rose, the average yield loss being 30.3% when CI was more than 70 (Fig. 1). However, this effect of stripe rust varied among locations. The average yield loss was over 50% in Eskisehir and Altıntas, while it was 23.4% in Sivasli.

## Discussion

The effect of stripe rust on wheat grain yields varies depending on genotypes and locations. This study involved a total of eight different RYTs in five locations, and one YT and PYT in each of two locations. The findings of the

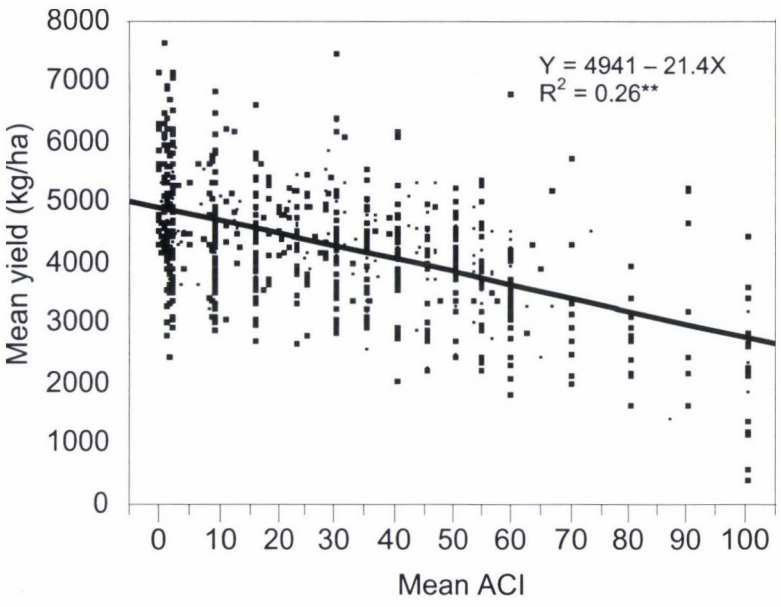



*Fig.1.* Effect of stripe rust on grain yields in Eskisehir (Y = grain yield, X = ACI)

study show great similarities to the results previously reported on the same topic (Kaidash et al., 1976; Cromey, 1989; Braun and Saari, 1992; Ketata, 2001; Finckh and Mundt, 1992; Duwadi et al., 1993; Chen et al., 2002; Ma and Singh, 1996). The most intensive epidemics were observed in the Altintas location (Table 1), and consequently the greatest yield loss occurred in this location (Table 2). By contrast, the lowest yield loss occurred in Sivaslı, where the epidemic was the least intensive. The Altintas location has the longest growing season among all the locations in the experiment, the harvest date being approximately 10 days later than at the main station in Eskisehir. On the other hand, Sivaslı has the shortest season, being approximately 2 weeks earlier as compared to Eskisehir. Banaz also had a longer season, resulting from the moist climate and lower average temperature due to its higher altitude. Previous research revealed that the earlier the epidemics start and the more suitable the climatic conditions are, the greater the yield reductions caused by stripe rust. Yield losses increase with longer duration of rust development (Murray et al., 1994; Park et al., 1988; Ash and Brown, 1990; Kaidash et al., 1976).

Besides climatic conditions, the source of inoculum and the susceptibility of the genotypes are also important in stripe rust development. The yield losses of the susceptible standards involved in the study are given for locations and trials in Table 3. The greatest yield loss was suffered by Gerek79, with 87.0% in the 1st RYT in Altıntas. This was followed by the 83.6% yield loss for the same variety in the 2nd RYT in Eskisehir. Kirgiz95 was the second most susceptible

genotype after Gerek79, with 72.2 and 58.7% yield losses in the 1st and 2nd RYT, respectively, in Altintas. These were followed by Gun91 with 52.7 and 47.4% yield losses in Banaz and Altintas, respectively, while Kutluk94 was the fourth most susceptible variety with 50.4 and 41.2% yield losses in Altıntas and Eskisehir. Ketata (2001) reported similar results, finding yield losses varying from 52 to 4% among eight genotypes. Similar findings were reported by Ma and Singh (1996), Wagoire et al. (1998), Sharma et al. (1985), Park and Welling (1992) and Ash and Brown (1990).

Yield losses calculated using the second method are presented in Tables 4, 5 and 6. In all trials, the results were found to be parallel to those from the first method. Yield losses increased with higher disease severity. ACI is a common expression of disease severity and host reaction. Hacke (1992), Murray et al. (1994), Ma and Singh (1996), Bryson et al. (1995) and Allan and Pritchett (1972) calculated yield losses using various methods.

Murray et al. (1994), King (1976), Schultz and Line (1992) and Cortazar (1984) reported a negative relationship between yield and stripe rust severity. The present study also revealed a linear relationship between yield and ACI (Fig. 1). The analysis, using a total of 697 lines and varieties, showed a negative correlation, implying significant increases in yield losses as ACIs rise.

The results of these evaluations, based on three different methods, show that serious yield losses occur in years of epidemics due to the susceptibility of widely grown cultivars. Although it is possible to control the disease through the use of chemicals (Darwinkel, 1980; Smith et al., 1986), it is not economically feasible under the present economic conditions. Developing resistant varieties is the main objective of the relevant breeding programmes. Except for very specific studies, ACI can be used as a criterion in selection from large populations. In the light of yield loss calculations, the elimination of lines with a coefficient of infection of over 30 will be helpful.

As to the comparison of the methods used in the calculations, the first method can be suggested for calculating yield losses of individual entries, the second method for selection in breeding programmes, and the third method for calculating yield losses in disease surveys.

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# QUALITY OF MAIZE (*Zea mays* L.) KERNELS AS AFFECTED BY THE NP SUPPLIES OF THE SOIL

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In a long-term mineral fertilisation experiment with 64 treatments representing all possible combinations of four rates each of N, P and K, set up on chernozem meadow soil in Szarvas in 1989, the protein and oil contents and the amino acid and fatty acid compositions of the maize grain yield were analysed between 1997 and 2004.

The protein content of the maize kernels increased by 1.1–1.5 percentage points up to a $NO_3$-N level of 80–100 kg $ha^{-1}$ in the 0–60 cm soil layer prior to sowing. The year had a greater influence on the protein content than the N supplies. No consistent effect of N on the amino acid composition, detectable as a change in the ratio of any amino acid in the majority of experimental years, was observed. In the AL-$P_2O_5$ range of 120–362 mg $kg^{-1}$ in the ploughed layer, the soil P supplies had no statistically significant effect on the kernel protein content. In most years the P supplies had little effect on the amino acid composition of the protein. The oil content and fatty acid composition of maize kernels was extremely stable, and was very little affected by the nutrient supplies or the year. During the experimental period excessive N supplies were only found to reduce the oil content and modify the fatty acid composition on one occasion. The oil content and fatty acid composition were not substantially affected by the P supplies.

**Key words**: maize, protein and oil content, amino and fatty acid composition, N and P fertiliser response

## Introduction

The biological value of maize fodder is fundamentally determined by the protein and oil content of the kernels and by the amino acid and fatty acid composition. The protein content of the normal maize varieties in general cultivation is around 8–12%. The feed value of maize kernel protein is relatively poor, as it consists mainly of zein (50–55%) and glutelin (30–45%), which are poor in tryptophan, lysine and methionine (Lásztity, 1981; Larkins et al., 1993;

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Matuz et al., 2000; Győri and Mile, 2002). Considerable differences can be observed between the protein contents and amino acid compositions in the various morphological parts of the kernel. The germ contains 17–20% protein, the endosperm 7–10% and the seedcoat 3–4%, but if the mass ratio is considered, 70–75% of the total protein is concentrated in the endosperm and 20–25% in the germ. As regards the amino acid composition, the proteins in the germ and the aleuron layer are richer in lysine. The storage proteins in the endosperm are particularly poor in lysine and tryptophan, but they have high glutamic acid, proline and leucine contents (El-Kady et al., 1983; Lásztity, 1999). Maize kernels have an oil content of 3–6% and the fatty acid composition is dominated by unsaturated linoleic acid (C 18:2) and oleic acid (C 18:1), which make up 40–60% and 25–40%, respectively. The germ has the highest oil content (30–35%), compared with only 0.7–1.0% in the endosperm. In terms of mass ratio, 80–85% of the total lipid is found in the germ (Patel and Sanghi, 1990; Lásztity, 1999; Győri and Mile, 2002). As the protein and oil are unevenly distributed in the kernels, any factors that cause a change in the kernel mass and in the mass ratio of kernel parts may influence the contents and composition of the protein and oil.

The protein content and amino acid composition of maize kernels are mainly determined genetically, but they may be modified by ecological and agronomic factors. Among the latter, the effect of nutrient supplies on quality is the most pronounced. It has been widely proved that better N supplies improve the protein content (Veress, 1973; Kovács, 1994; Prokszáné et al., 1995; Izsáki, 1999; Kramarev et al., 2000). The correlation between N supplies and the amino acid composition of the protein is less consistent. Investigations by Veress (1973) indicated that N fertilisation caused an increase in the quantity of most amino acids, but the rate of increase in essential amino acids was less than for non-essential amino acids. Experiments reported by Németh (1985) demonstrated that up to a rate of 261 kg $ha^{-1}$, N fertilisation only improved the essential amino acid index to a slight extent. N fertilisation reduced the proportion of methionine and leucine in the protein, had little effect on that of lysine, but increased that of the other essential amino acids. The relative stability of limiting essential amino acids was confirmed by the data of Győri and Mile (2002), who found no significant change in the quantities of lysine, methionine and cystine within the total protein at N fertiliser rates up to 240 kg $ha^{-1}$. On soil with satisfactory N supplies, Kádár (2000) was unable to detect any significant influence of N fertilisation on the protein content or the amino acid composition. At the same time, increasing soil P supplies, up to a rate of 189 mg $kg^{-1}$ $P_2O_5$, improved the ratio of essential amino acids. Németh (1985) also reported that better P supplies resulted in a more favourable essential amino acid index and that P fertilisation caused an increase in the ratio of the majority of essential amino acids, only reducing the quantities of isoleucine and leucine.

The effect of fertilisation on protein content and the amino acid composition may be modified by the year, especially the water supplies. Results published by Prokszáné et al. (1995) showed that the protein content of maize was smaller in wet years than in dry years, both without N fertilisation and at various N fertiliser levels. Irrigation also caused a decrease in the protein content, particularly at high stand densities (Prokszáné and Harmati, 1988). In dry years mineral fertilisation resulted in a greater increase in the crude protein content (Győri and Mile, 2002; Prokszáné et al., 1995). The year may influence the quantity of certain amino acids, but others have stable values over the years (Pásztor et al., 1997; 1998).

Results achieved in both Hungary and abroad suggest that the oil content and fatty acid composition of maize kernels exhibit relatively high stability, and neither nutrient supplies nor the year have any substantial influence on these quality parameters. Using a 60-year data series, Earle (1977) found no correlation between the oil content and either temperature/rainfall or fertilisation. Prokszáné et al. (1995), Pásztor et al. (1998) and Gyenesné et al. (2001) were unable to detect a statistically significant year effect on the oil content. According to Prokszáné et al. (1995) high rates of N fertiliser (200–300 kg N $ha^{-1}$) caused a significant decline in the oil content of maize hybrids, but these small deviations could not be meaningfully evaluated. The year, particularly the temperature, was reported by Hilditch and Williams (1964) and Hitchcock and Nichols (1971) to have an influence on the fatty acid composition.

Over the last decade, the need for precise, environment-friendly, quality-oriented plant nutrition for maize has made it imperative to place N fertilisation on new foundations, by elaborating and implementing a fertilisation system based on the measurement of soil mineral N contents (Hoffmann, 1993; Németh, 1996). In connection with this N fertilisation system, few research results are available on the correlation between the $N_{min}$ reserves of the soil prior to sowing and the quality of maize. A knowledge of correlations between soil P supplies and maize quality for various soil types is equally important if a P fertilisation system is to be elaborated for maize. The present paper provides further results that can be used for the development of quality-oriented N and P fertiliser recommendations.

## Materials and methods

Long-term mineral fertilisation experiments were set up at the Experimental Station of the Crop Production Department, Faculty of Agricultural Water and Environmental Management, Tessedik Sámuel College, Szarvas, in 1989. The soil of the experimental area had the following parameters: chernozem meadow soil, calcareous in the deeper layers, 85–100 cm humus layer, $pH_{(KCl)}$ 5.0–5.2, humus content 3.0–3.2%, upper limit of plasticity according to Arany ($K_A$) 50, clay content 32%. Prior to the setting up of the experiment the soil contained the following nutrient supplies: AL-$P_2O_5$ 156 mg $kg^{-1}$, AL-$K_2O$ 322 mg $kg^{-1}$, AL-Na 212 mg $kg^{-1}$, KCl-Mg 765 mg $kg^{-1}$, EDTA-Mn 386 mg $kg^{-1}$, EDTA-Cu 5.4 mg $kg^{-1}$ and EDTA-Zn 3.0 mg $kg^{-1}$.

Fertilisation was carried out in all possible combinations of four levels each of N, P and K, giving a total of 64 treatments, set up in a split-split plot design with three replications, with K fertilisation as the "A" factor, P fertilisation as the "B" factor and N fertilisation as the "C" factor. The following fertiliser rates were applied: nitrogen: $N_0 = 0$, $N_1 = 80$, $N_2 = 160$, $N_3 = 240$ kg N $ha^{-1}$ $year^{-1}$; phosphorus ($P_2O_5$): $P_0 = 0$, $P_1 = 100$ kg $ha^{-1}$ $year^{-1}$, $P_2 = 500$ kg $ha^{-1}$ in 1989, 1993 and 2001, $P_3 = 1000$ kg $ha^{-1}$ in 1989, 1993 and 2001; potassium ($K_2O$): $K_0 = 0$, $K_1 = 300$ kg $ha^{-1}$ $year^{-1}$ between 1989 and 1992, 100 kg $ha^{-1}$ $year^{-1}$ from 1993, $K_2 = 600$ kg $ha^{-1}$ in 1989 and 2001, 1000 kg $ha^{-1}$ in 1993, $K_3 = 1200$ kg $ha^{-1}$ in 1989 and 2001, 1500 kg $ha^{-1}$ in 1993. The high rates of P and K replenishment fertilisation were used to create clearly distinct supply levels in the soil in order to investigate plant responses to nutrient status. The nitrogen was applied as ammonium nitrate, the phosphorus as superphosphate and the potassium as potassium chloride in autumn. In each year of the experiment, four crops were included in the crop rotation on 4 × 192 plots, where the plot size of the sub-sub-plots was $4 \times 5 = 20$ $m^2$.

In order to determine the level of soil nutrient supplies, soil samples were taken from the 0–60 cm soil level each year in autumn after harvesting the forecrop to analyse the P and K contents with the AL (0.1 M $NH_4$-lactate + 0.4 M acetic acid) method. When evaluating the results the soil P and K supply levels are given as the AL-$P_2O_5$ and $K_2O$ contents of the ploughed layer. The mineral nitrogen content of the soil in the 0–60 cm soil layer was determined in spring, prior to sowing. The $NO_3$-N + $NH_4$-N content determined using the 1 N KCl method is given in the paper as an estimation of the N supply level. The nitrogen and phosphorus supplies of the soil during the experimental period are presented in Table 1.

For the calculation of crude protein content (N×6.25) the total N was measured by the macro-Kjeldahl method. The amino acids were measured after acidic (6 N HCl) hydrolysis by ion exchange column chromatography (HPLC, Aminochrom II. Labor, MIM Hungary). The total fatty acids were extracted, methyl-esterified and analysed by gas-liquid chromatography (GLC, UNICAM PRO-GC).

The maize experiments have been underway since 1994. The present paper presents the results of trials carried out on the hybrid Clarica (FAO 310, Pioneer) between 1997 and 2004. The forecrop was fibre hemp from 1997–1999 and silage sorghum between 2000 and 2004. Sowing was carried out with a row distance of 75 cm and 75,000 germs $ha^{-1}$.

Data on the rainfall supplies in the various years, characterised by the quantity of rain up to the beginning of tasselling and over the whole vegetation period, demonstrate that the years with the best rainfall distribution and supplies were 1997, 1999 and 2001. In 1998, 2002 and 2004 the rainfall distribution was less favourable, but total rainfall over the growing season exceeded the many years' mean, while 2000 and 2003 were dry or droughty years (Table 2). The mean temperature during the growing season was 18.5, 19.5, 20.4 and 18.0°C, while the many years' mean was 18.0°C.

## Results and discussion

The mineral N content of the upper 0–60 cm soil layer prior to sowing ($N_{min}$) is illustrated in the tables as the quantity of $NO_3$-N and $NH_4$-N at various N fertiliser levels. In agreement with earlier studies (Németh and Búzás, 1984; Hoffmann, 1993) the results indicate that the $NH_4$-N content was not correlated with the N fertiliser level and was relatively low in most years (10–30 kg $ha^{-1}$). A slightly higher $NH_4$-N content (40–60 kg $ha^{-1}$) was recorded in years when the soil was poorly aerated in early spring due to long-term snow cover or water saturation (1997, 2003). By comparison, the $NO_3$-N content of the soil increased with rising N fertiliser rates (Table 1). Consequently, the soil N supplies were characterised chiefly by the soil $NO_3$-N content when evaluating the results.

*Table 1*
Nitrogen ($NO_3$+NH4.N kg $ha^{-1}$ in 0–60 cm soil layer) and phosphorus (AL-$P_2O_5$ mg $kg^{-1}$ in cultivated layer) supplies of the soil during the experimental period (Szarvas, 1997–2004)

| Year | $N_0$ | $N_1$ | $N_2$ | $N_3$ |
|---|---|---|---|---|
| 1997 | 58+36 | 100+41 | 138+40 | 205+53 |
| 1999 | 31+17 | 40+20 | 80+18 | 86+18 |
| 2000 | 21+28 | 20+30+80$^+$ | 22+32+160$^+$ | 22+28+240$^+$ |
| 2001 | 38+5 | 80+6 | 131+8 | 148+11 |
| 2002 | 60+18 | 85+19 | 180+20 | 205+21 |
| 2003 | 39+40 | 55+51 | 61+46 | 77+61 |
| 2004 | 64+22 | 96+22 | 133+23 | 191+22 |
| | $P_0$ | $P_1$ | $P_2$ | $P_3$ |
| 1997 | 155 | 194 | 233 | 283 |
| 1999 | 158 | 175 | 217 | 267 |
| 2000 | 138 | 194 | 185 | 239 |
| 2001 | 120 | 183 | 156 | 204 |
| 2002 | 120 | 176 | 153 | 1200 |
| 2003 | 128 | 183 | 195 | 339 |
| 2004 | 139 | 198 | 222 | 362 |

+ Ammonium nitrate applied in spring; $N_0 = 0$, $N_1 = 80$, $N_2 = 160$, $N_3 = 240$ kg N $ha^{-1}$; $P_0 = 0$, $P_1 = 100$ kg $ha^{-1}$ $year^{-1}$, $P_2 = 500$ kg $ha^{-1}$ in 1989, 1993 and 2001, $P_3 = 1000$ kg $ha^{-1}$ in 1989, 1993 and 2001

*Table 2*
Rainfall quantities and distribution during the experimental years, mm (Szarvas, 1997–2004)

| Year | Up to the beginning of tasselling (Apr.–Jun.) | During the growing season (Apr.–Aug.) | Outside the growing season (Sep.–Mar.) | Annual total |
|---|---|---|---|---|
| 1996 | 170 | 386 | 348 | 663 |
| 1997 | 174 | 309 | 231 | 489 |
| 1998 | 151 | 330 | 193 | 606 |
| 1999 | 296 | 446 | 330 | 847 |
| 2000 | 80 | 152 | 341 | 339 |
| 2001 | 204 | 337 | 254 | 612 |
| 2002 | 124 | 303 | 196 | 489 |
| 2003 | 29 | 77 | 262 | 350 |
| 2004 | 164 | 356 | 271 | 659 |
| Average* | 171 | 274 | 264 | 538 |

*Long-term average (1901–1975)

Under the given experimental conditions, the K supplies had no influence on the protein and oil content or amino acid and fatty acid composition of maize kernels over an AL-$K_2O$ range of 272–490 mg $kg^{-1}$ in the ploughed layer. No interaction between the effects of N and P supplies on the protein content and amino acid composition was detected, so only the main effects of N and P are discussed. In the case of the oil content and fatty acid composition only the N supplies had a slight effect, so only these results are presented.

*1. Protein content*

The protein content of maize kernels ranged from 7.3–12.9% during the experimental years, as a function of N supplies and year. An evaluation of the N effect shows that in most years the protein content rose significantly by 1.1–1.5 percentage points compared to the N control (given no N fertiliser) as the $NO_3$-N level in the 0–60 cm soil layer prior to sowing increased to 80–100 kg $ha^{-1}$, achieved with N fertilisation of 80 kg $ha^{-1}$. A further slight increase in the protein content was detected when the $NO_3$-N reserves in the soil prior to sowing reached 120–140 kg $ha^{-1}$. At higher N supply levels no further change in the protein content was observed (Table 3, Figs. 1 and 2).

*Table 3*
Effect of N supplies on the protein content (g 100 $g^{-1}$ dry matter) of maize kernels (Szarvas, 1997–2004)

| Year | $N_0$ | $N_1$ | $N_2$ | $N_3$ | $LSD_{5\%}^{++}$ | Average |
|---|---|---|---|---|---|---|
| 1997 | 7.30 | 8.40 | 8.60 | 8.80 | 0.30 | 8.24 |
| 1999 | 7.70 | 7.99 | 8.73 | 8.97 | 0.62 | 8.34 |
| 2000 | 9.37 | 10.10 | 10.41 | 10.25 | 0.51 | 10.03 |
| 2001 | 8.44 | 9.65 | 10.02 | 10.18 | 0.67 | 9.57 |
| 2002 | 11.17 | 12.43 | 12.74 | 12.87 | 0.54 | 12.30 |
| 2003 | 10.22 | 11.39 | 11.54 | 11.76 | 0.76 | 11.22 |
| 2004 | 10.05 | 11.57 | 12.02 | 11.91 | 0.62 | 11.39 |
| $LSD_{5\%}^{+}$ | 0.75 | 0.46 | 0.38 | 0.56 | – | 1.05 |
| Range‡ | 7.30–11.17 | 7.99–12.43 | 8.60–12.74 | 8.80–12.87 | | 8.27–12.30 |

+between years; ++between treatments; ‡of protein content; $N_0 = 0$, $N_1 = 80$, $N_2 = 160$, $N_3 = 240$ kg N $ha^{-1}$

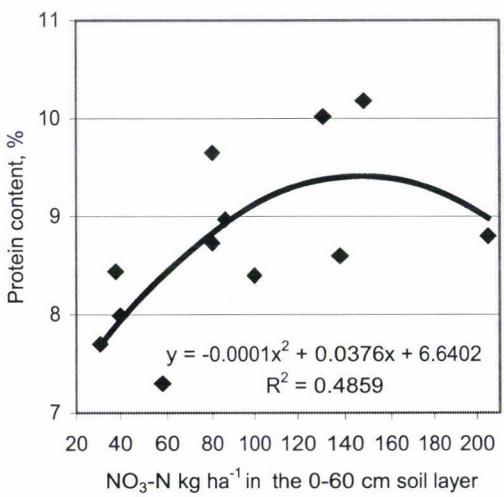



*Fig. 1.* Relationship between the $NO_3$-N content of the soil prior to sowing and the protein content of maize kernels in years with high grain yields (Szarvas, 1997, 1999, 2001)

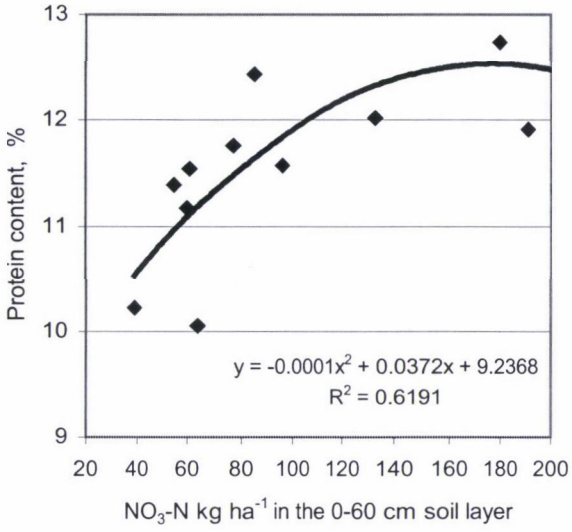



*Fig. 2.* Relationship between the $NO_3$-N content of the soil prior to sowing and the protein content of maize kernels in years with low grain yields (Szarvas, 2002, 2003, 2004)

The year had a greater effect on the protein content of maize kernels than the N supplies. While N fertilisation increased the protein content by a maximum of 1.3–2.0 percentage points, the effect of the year was 2–3 times as great. In long-term fertilisation experiments carried out by Kovács (1994) even greater year effects were reported for protein content, 6–7 times that of the nutrient supply level. When the year effect was analysed for various N supply levels, the fluctuation in the protein content over the experimental period was found to be 3.9% in the N control and 4.1–4.4% in the N treatments, indicating that better N supplies did not reduce the changes in protein content resulting from the year effect. When the effect of N supplies was evaluated for each year it could be seen that in years with poor rainfall supplies N fertilisation resulted in a slightly (0.2–0.3 percentage points) higher protein content compared with years with good water supplies (Table 3).

As the year had a greater modifying effect on protein content than the N supplies, the experimental years were grouped according to yield level when evaluating the year effect. Years were considered to have a high yield when the grain yield achieved on soil with an 80–100 kg $ha^{-1}$ $NO_3$-N content in the 0–60 cm layer prior to sowing exceeded 8.5 t $ha^{-1}$. Years when the grain yield at this N supply level was less than 7.5 t $ha^{-1}$ were considered to have low yields. The close correlation between rainfall distribution, the water supplies during the vegetation period and the yield has been confirmed by many authors (Nagy and Huzsvai, 1996; Huzsvai and Nagy, 2003; Berzsenyi and Lap, 2002; 2003). Under the present experimental conditions too, years with high yields had more favourable rainfall distribution and water supplies than those giving lower

yields. A negative correlation between yield and protein content was reported by Kralovánszky (1975) and Bálint (1977). In the present experiment it was found that in years with high yields the protein content, averaged over the N treatments, was lower (8.3–10%) than in years with poorer yields, when it ranged from 11.2–12.3%. However, even in this grouping a smaller, but significant year effect could be detected. The correlation between soil $NO_3$-N supplies and grain protein content was weak to medium (r=0.41) if years with different weather conditions were analysed together. When the years were grouped according to yield level, however, the correlation between N supplies and protein content was closer, with values of r=0.70 in high-yielding years and r=0.79 in years with lower yields (Table 3, Figs. 1, 2 and 3).

No significant change in the protein content of maize kernels was observed over an AL-$P_2O_5$ supply range of 120–362 mg $kg^{-1}$ in the ploughed layer.

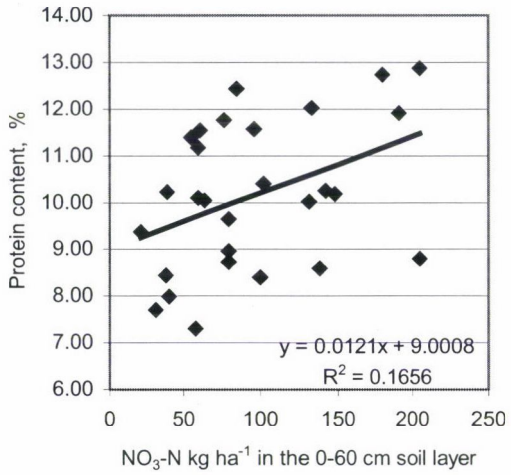



*Fig. 3.* Relationship between the $NO_3$-N content of the soil prior to sowing and the protein content of maize kernels (Szarvas, 1997–2004)

*2. Amino acid composition*

*2.1. Effect of N supplies*

As regards the amino acid composition of the protein (g 100 $g^{-1}$ protein), none of the amino acids exhibited a consistent change in ratio in all the experimental years as the result of N treatment. Of the 17 amino acids examined, Table 4 lists only those that responded to N supplies with a significant change. The N supplies had no effect on the ratio of isoleucine, threonine, alanine, cysteine, glutamic acid or proline in the protein. Of the seven years investigated, the leucine ratio increased in three years, while that of lysine declined at higher N supply levels. In two experimental years there was a significant increase in phenylalanine, but a decrease in the glycine ratio as the result of increasing N supplies. Arginine, methionine, valine, aspartic acid, serine and tyrosine

exhibited significant but inconsistent increases or decreases in two or three years as a function of N supplies. The ratio of essential amino acids exhibited a slight increase in most years up to the 80–100 kg $ha^{-1}$ $NO_3$-N supply level.

It can be concluded from the data that on this chernozem meadow soil, which had a humus content of almost 3% and which, according to previous studies (Izsáki and Iványi, 2005), was able to provide around 100 kg $ha^{-1}$ N each year without N fertilisation, the application of an 80 kg $ha^{-1}$ rate of N fertilisation, resulting in 80–100 kg $ha^{-1}$ $NO_3$-N content in the upper 0–60 cm soil layer prior to sowing, produced a near maximum grain yield (Izsáki, 1999; 2003) and favourable protein content and amino acid composition, which could not be significantly improved by higher N supply levels.

*Table 4*

Effect of N supplies in the 0–60 cm soil layer on the amino acid composition (amino acid g 100 $g^{-1}$ protein ) of maize protein (Szarvas, 1997–2004)

| Amino acid | $N_0$ | $N_1$ | $N_2$ | $N_3$ | $LSD_{5\%}$ |
|---|---|---|---|---|---|
| 1997 | | | | | |
| Phenylalanine | 3.85 | 4.40 | 4.44 | 4.33 | 0.42 |
| Leucine | 9.90 | 10.71 | 10.52 | 11.17 | 0.82 |
| Methionine | 1.78 | 1.54 | 1.40 | 1.59 | 0.21 |
| Glutamine | 16.23 | 16.42 | 17.19 | 18.24 | 1.34 |
| Serine | 4.95 | 5.11 | 5.14 | 5.47 | 0.31 |
| 1999 | | | | | |
| Lysine | 5.83 | 5.02 | 4.68 | 4.89 | 0.97 |
| Methionine | 1.68 | 1.87 | 1.48 | 1.55 | 0.25 |
| Glycine | 4.73 | 4.66 | 4.23 | 4.21 | 0.47 |
| Tyrosine | 1.95 | 1.93 | 1.71 | 2.47 | 0.41 |
| 2000 | | | | | |
| Glycine | 3.97 | 3.86 | 3.64 | 3.77 | 0.26 |
| 2001 | | | | | |
| Leucine | 10.90 | 11.60 | 12.37 | 12.08 | 1.18 |
| Lysine | 4.02 | 3.62 | 3.29 | 3.33 | 0.65 |
| Valine | 4.38 | 4.14 | 4.19 | 3.73 | 0.58 |
| Aspartic acid | 8.17 | 6.83 | 7.48 | 7.17 | 0.65 |
| Serine | 5.09 | 4.76 | 4.89 | 4.42 | 0.46 |
| Tyrosine | 2.84 | 2.38 | 2.59 | 2.45 | 0.23 |
| 2002 | | | | | |
| Leucine | 12.71 | 13.43 | 13.26 | 13.59 | 0.45 |
| Lysine | 3.84 | 3.53 | 3.29 | 3.65 | 0.32 |
| 2003 | | | | | |
| Arginine | 4.28 | 4.73 | 5.16 | 4.23 | 0.75 |
| Phenylalanine | 5.16 | 5.22 | 5.52 | 5.72 | 0.53 |
| Histidine | 2.74 | 2.73 | 3.04 | 3.17 | 0.42 |
| Methionine | 2.30 | 2.43 | 2.48 | 2.04 | 0.14 |
| Valine | 4.55 | 4.93 | 4.97 | 5.05 | 0.45 |
| Tyrosine | 2.89 | 3.79 | 3.83 | 2.54 | 0.61 |
| 2004 | | | | | |
| Arginine | 4.40 | 3.31 | 3.06 | 3.69 | 0.53 |
| Aspartic acid | 7.60 | 7.23 | 8.17 | 7.21 | 0.82 |
| Serine | 4.71 | 4.88 | 5.40 | 4.70 | 0.68 |

$N_0 = 0$, $N_1 = 80$, $N_2 = 160$, $N_3 = 240$ kg N $ha^{-1}$

### *2.2. Effect of P supplies*

Over a range of 120–362 mg $kg^{-1}$ AL-$P_2O_5$ the P supplies in the ploughed layer did not influence the protein content of maize kernels. Slight changes were observed, however, in the amino acid content of the protein in response to P supplies. Of the 17 amino acids investigated, only those where a significant P effect was detected in at least one experimental year are included in Table 5. The ratio of phenylalanine, leucine, methionine and serine within the protein was not influenced by the P supplies in any of the years. There was an increase in the arginine ratio in three of the seven experimental years and in that of isoleucine in two years, while the ratio of valine and aspartic acid declined in response to P fertilisation. The changes in these amino acid ratios within the protein could not be associated consistently with a similar P supply level in the various years.

*Table 5*

Effect of P supplies in cultivated layer on the amino acid composition (amino acid g $100g^{-1}$ protein) of maize protein (Szarvas, 1997–2004)

| Amino acid | $P_0$ | $P_1$ | $P_2$ | $P_3$ | $LSD_{5\%}$ |
|---|---|---|---|---|---|
| 1997 | | | | | |
| Arginine | 3.60 | 3.89 | 4.02 | 4.12 | 0.40 |
| Threonine | 3.72 | 3.52 | 4.24 | 4.24 | 0.41 |
| Proline | 11.05 | 9.61 | 7.27 | 7.27 | 1.21 |
| Tyrosine | 1.92 | 2.43 | 1.93 | 1.93 | 0.53 |
| 1999 | | | | | |
| Arginine | 4.54 | 4.08 | 4.52 | 5.70 | 1.05 |
| Histidine | 3.71 | 3.00 | 2.96 | 4.13 | 0.59 |
| Isoleucine | 3.23 | 3.09 | 3.38 | 3.52 | 0.24 |
| Lysine | 5.18 | 4.48 | 4.97 | 5.79 | 0.92 |
| Cysteine | 1.57 | 1.42 | 1.80 | 1.70 | 0.25 |
| Glycine | 4.53 | 4.20 | 4.33 | 4.76 | 0.51 |
| 2000 | | | | | |
| Valine | 5.27 | 4.88 | 4.73 | 5.04 | 0.31 |
| Aspartic acid | 8.82 | 8.32 | 7.79 | 8.08 | 0.52 |
| Cysteine | 1.35 | 1.35 | 1.23 | 0.96 | 0.29 |
| Glutamic acid | 18.00 | 16.55 | 17.12 | 17.20 | 1.52 |
| 2001 | | | | | |
| Arginine | 4.62 | 5.40 | 5.51 | 4.16 | 0.73 |
| Histidine | 3.22 | 3.83 | 4.19 | 3.32 | 0.67 |
| 2003 | | | | | |
| Isoleucine | 3.37 | 3.88 | 3.39 | 3.35 | 0.21 |
| Proline | 5.96 | 7.40 | 7.50 | 6.60 | 1.29 |
| 2004 | | | | | |
| Arginine | 4.40 | 3.31 | 3.06 | 3.69 | 0.53 |
| Lysine | 2.21 | 2.73 | 2.29 | 1.96 | 0.63 |
| Threonine | 3.80 | 4.10 | 3.81 | 3.46 | 0.52 |
| Valine | 4.92 | 5.47 | 4.88 | 4.34 | 0.77 |
| Alanine | 8.91 | 10.60 | 9.52 | 8.30 | 1.17 |
| Aspartic acid | 7.57 | 7.95 | 7.78 | 6.91 | 0.90 |
| Serine | 4.81 | 5.34 | 5.00 | 4.54 | 0.67 |

$P_0$=0, $P_1$=100 kg $ha^{-1}$ $year^{-1}$, $P_2$= 500 kg $ha^{-1}$ in 1989, 1993 and 2001, $P_3$= 1000 kg $ha^{-1}$ in 1989, 1993 and 2001

Changes of varying sign were observed on 2–3 occasions each for histidine, lysine, threonine, cysteine and proline at various P supply levels. In one year a significant rise in the ratio of alanine, glycine and tyrosine was observed, with a reduction in the glutamic acid ratio as the P supply level increased. The ratio of essential to non-essential amino acids in the protein was not influenced by P supplies.

It can be seen from the data that the amino acid composition of the maize kernel protein only changed to a slight extent over an AL-$P_2O_5$ range of 120–362 mg $kg^{-1}$ in the ploughed layer, while the ratio of the individual amino acids was not influenced by the P supplies in most years.

*2.3. Effect of the year*

The trends in the amino acid composition of maize kernel protein in the experimental years, averaged over the fertiliser treatments, can be seen in Table 6.

In most years the ratio of individual amino acids in the protein was not influenced by N and P supplies. By contrast, the effect of the year on the amino acid composition was very pronounced. Over the seven experimental years the ratio of individual amino acids fluctuated over a fairly wide range, and significant differences could be demonstrated between the years for all the amino acids in most cases. The ratio of lysine, methionine and cysteine, the group of amino acids that is limiting for the fodder value of maize, also varied greatly, from 5.64–8.37%. The ratio of essential amino acids ranged from 41–45% over the experimental period. The annual changes experienced in the amino acid composition were not consistently correlated either with the protein content or with the yield. The explanation of the year effect will require further analysis.

*3. Oil content and fatty acid composition*

Depending on the N supplies, the oil content of the maize kernels varied over a narrow range (4.21–4.76%) during the experimental period. Only in one of the four years (2004) was there a significant, but still not large reduction in oil content at the highest N supply level. The year had a slight modifying effect on the oil content, averaged over the N treatments, which was occasionally significant. However, this change in the oil content could not be explained by either the water supplies or the temperature during the grain-filling period or over the whole vegetation period. The results did not confirm the findings of Veneni (1971) who reported a decline in the kernel oil content in cooler than average years (Table 7).

These results suggest that the oil content of the kernel is extremely stable, being only slightly influenced by nutrient supplies and the year.

The fatty acid composition was only significantly influenced by the N supplies in one year (2004), when the oleic acid content dropped and that of linoleic acid rose when the $NO_3$-N level in the 0–60 cm soil layer prior to sowing was 96 kg $ha^{-1}$. However, these changes were relatively slight. At higher N supply levels no further significant changes could be detected (Table 7).

*Table 6*
Amino acid composition of maize kernels during the experimental years, g 100 $g^{-1}$ protein (Szarvas, 1997–2004)

| Amino acid (AA) | HGY* | | | LGY** | | | | $LSD_{5\%}$ | Aver-age | Average | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1997 | 1999 | 2001 | 2000 | 2002 | 2003 | 2004 | | | HGY | LGY |
| Essential AA | | | | | | | | | | | |
| Arginine | 3.87 | 4.71 | 4.93 | 4.22 | 3.83 | 4.60 | 3.62 | 0.52 | 4.25 | 4.50 | 4.06 |
| Phenylalanine | 4.26 | 5.13 | 5.38 | 5.09 | 5.01 | 5.40 | 4.92 | 0.34 | 5.02 | 4.92 | 5.10 |
| Histidine | 2.65 | 3.45 | 3.64 | 2.87 | 3.16 | 2.92 | 3.12 | 0.34 | 3.12 | 3.24 | 3.01 |
| Isoleucine | 2.90 | 3.31 | 3.09 | 3.50 | 3.26 | 3.49 | 3.97 | 0.21 | 3.36 | 3.10 | 3.55 |
| Leucine | 10.58 | 10.89 | 11.73 | 11.43 | 13.24 | 11.92 | 11.30 | 0.65 | 11.58 | 11.06 | 11.97 |
| Lysine | 3.79 | 5.10 | 3.56 | 4.01 | 3.57 | 3.61 | 2.30 | 0.44 | 3.71 | 4.15 | 3.57 |
| Methionine | 1.57 | 1.65 | 0.99 | 0.93 | 2.15 | 2.31 | 2.16 | 0.19 | 1.68 | 1.40 | 1.88 |
| Threonine | 3.84 | 3.91 | 3.79 | 3.63 | 3.77 | 3.29 | 3.79 | 0.20 | 3.72 | 3.84 | 3.62 |
| Valine | 5.25 | 4.85 | 4.11 | 4.98 | 3.73 | 4.87 | 4.90 | 0.39 | 4.67 | 4.73 | 4.62 |
| Total EAA | 38.71 | 43.00 | 41.22 | 40.66 | 41.72 | 42.41 | 40.08 | – | 41.11 | 40.94 | 41.18 |
| Non-essential AA | | | | | | | | | | | |
| Alanine | 7.02 | 7.72 | 7.09 | 7.55 | 7.97 | 7.77 | 9.33 | 0.37 | 7.78 | 7.23 | 8.16 |
| Aspartic acid | 6.69 | 7.96 | 7.41 | 8.25 | 7.02 | 6.72 | 7.55 | 0.45 | 7.37 | 7.35 | 7.39 |
| Cysteine | 1.82 | 1.62 | 1.09 | 1.22 | 2.09 | 1.96 | 2.27 | 0.16 | 1.72 | 1.51 | 1.88 |
| Glycine | 4.70 | 4.45 | 4.47 | 3.81 | 4.50 | 3.40 | 3.99 | 0.29 | 4.19 | 4.54 | 3.93 |
| Glutamic acid | 17.02 | 17.47 | 17.44 | 17.22 | 20.58 | 17.56 | 17.90 | 0.98 | 17.88 | 17.31 | 18.32 |
| Proline | 9.03 | 9.31 | 7.01 | 8.53 | 8.61 | 6.86 | 9.47 | 0.75 | 8.40 | 8.45 | 8.37 |
| Serine | 5.17 | 4.81 | 4.79 | 4.88 | 5.13 | 4.59 | 4.92 | 0.29 | 4.90 | 4.92 | 4.88 |
| Tyrosine | 2.15 | 2.01 | 2.56 | 2.95 | 0.83 | 3.26 | 2.48 | 0.43 | 2.32 | 2.24 | 2.38 |
| Total NEAA | 53.60 | 55.35 | 51.86 | 54.41 | 56.73 | 52.12 | 57.91 | – | 54.56 | 53.55 | 55.31 |
| Total EAA+NEAA | 92.31 | 98.35 | 93.08 | 95.07 | 98.45 | 94.53 | 97.99 | – | 95.67 | 94.49 | 96.49 |
| EAA/NEAA | 42/58 | 44/56 | 44/56 | 43/57 | 42/58 | 45/55 | 41/59 | – | 43/57 | 43/57 | 43/57 |

* Years with high grain yield; ** Years with low grain yield

It has long been known that the composition of plant oils changes as a function of the temperature during the growing period. In cooler years there is an increase in the degree of unsaturation of the fatty acids (Hitchcock and Nichols, 1971). According to Hilditch and Williams (1964) these changes primarily affect the oleic, linoleic and linolenic acids, with a greater accumulation of oleic acid under warmer conditions at the expense of linoleic and linolenic acids.

The present results for maize did not confirm these findings, since the oleic acid content was significantly higher and the linoleic acid content significantly lower in 2001, when the mean temperature during the vegetation period was 18.5°C, than in 2002 and 2003, when the mean temperature was 19.5 and 20.4°C, respectively. The joint ratio of oleic and linoleic acid within the total lipids was practically unchanged, at 84.4–85% over the experimental period (Table 7). A negative correlation was found between the relative quantities of linoleic and oleic acid (Cheesbrough et al., 1997; Seo et al., 1998).

*Table 7*
Effect of N supplies in the 0–60 cm soil layer on the oil content and fatty acid composition of maize kernels, % (Szarvas, 2001–2004)

| Components | $N_0$ | $N_1$ | $N_2$ | $N_3$ | $LSD_{5\%}$ | Average |
|---|---|---|---|---|---|---|
| 2001 | | | | | | |
| Oil content, % | 4.21 | 4.26 | 4.36 | 4.34 | NS | 4.29 |
| Palmitic (C 16:0) | 11.72 | 11.60 | 11.46 | 11.67 | NS | 11.61 |
| Stearic (C 18:0) | 2.21 | 2.08 | 2.00 | 1.95 | NS | 2.06 |
| Oleic (C 18:1) | 37.39 | 37.10 | 36.99 | 37.12 | NS | 37.15 |
| Linoleic (C 18:2) | 47.14 | 47.63 | 47.94 | 47.89 | NS | 47.65 |
| Alpha-linolenic (C 18:3) | 1.07 | 1.09 | 1.15 | 0.89 | NS | 1.05 |
| Arachidic (C 20:0) | 0.49 | 0.47 | 0.47 | 0.41 | NS | 0.46 |
| 2002 | | | | | | |
| Oil content, % | 4.45 | 4.27 | 4.37 | 4.29 | NS | 4.35 |
| Palmitic (C 16:0) | 11.53 | 11.58 | 11.60 | 11.61 | NS | 11.58 |
| Stearic (C 18:0) | 1.85 | 1.82 | 1.81 | 1.79 | NS | 1.82 |
| Oleic (C 18:1) | 33.94 | 33.33 | 33.60 | 33.52 | NS | 33.60 |
| Linoleic (C 18:2) | 50.92 | 51.69 | 51.56 | 51.44 | NS | 51.40 |
| Alpha-linolenic (C 18:3) | 1.06 | 1.11 | 1.11 | 1.09 | NS | 1.09 |
| Arachidic (C 20:0) | 0.45 | 0.39 | 0.31 | 0.53 | NS | 0.42 |
| 2003 | | | | | | |
| Oil content, % | 4.58 | 4.60 | 4.54 | 4.55 | NS | 4.57 |
| Palmitic (C 16:0) | 11.94 | 11.91 | 11.74 | 11.97 | NS | 11.89 |
| Stearic (C 18:0) | 2.13 | 2.16 | 2.11 | 2.20 | NS | 2.15 |
| Oleic (C 18:1) | 34.57 | 34.75 | 34.53 | 34.76 | NS | 34.65 |
| Linoleic (C 18:2) | 49.75 | 49.62 | 50.05 | 49.49 | NS | 49.73 |
| Alpha-linolenic (C 18:3) | 1.05 | 1.04 | 1.05 | 1.05 | NS | 1.05 |
| Arachidic (C 20:0) | 0.56 | 0.52 | 0.52 | 0.53 | NS | 0.53 |
| 2004 | | | | | | |
| Oil content, % | 4.76 | 4.60 | 4.57 | 4.54 | 0.22 | 4.61 |
| Palmitic (C 16:0) | 11.45 | 11.63 | 11.59 | 11.42 | NS | 11.52 |
| Stearic (C 18:0) | 2.04 | 2.07 | 2.01 | 1.99 | NS | 2.03 |
| Oleic (C 18:1) | 35.52 | 34.52 | 34.34 | 34.34 | 0.91 | 34.68 |
| Linoleic (C 18:2) | 49.47 | 50.29 | 50.55 | 50.75 | 0.86 | 50.26 |
| Alpha-linolenic (C 18:3) | 1.02 | 0.99 | 1.00 | 1.02 | NS | 1.01 |
| Arachidic (C 20:0) | 0.49 | 0.49 | 0.51 | 0.48 | NS | 0.49 |

$LSD_{5\%}$ between years: Oil content: 0.11; Oleic acid: 0.50; Palmitic acid: 0.15; Linoleic acid: 0.60; Stearic acid: 0.09; NS=non-significant

## Conclusions

1. The protein content of maize kernels rose substantially, by 1.1–1.5 percentage points, when the $NO_3$-N level in the 0–60 cm soil layer rose to 80–100 kg $ha^{-1}$ prior to sowing. The year had a greater influence on the protein content than the N supplies.

2. A consistent N effect, detected as a change in the ratio of any amino acid in the majority of the experimental years, was not observed in the amino acid composition of the protein.

3. The P supplies had no statistically significant effect on the kernel protein content over an AL-$P_2O_5$ range of 120–362 mg $kg^{-1}$ in the ploughed layer. In most years the P supplies did not substantially influence the amino acid composition of the protein.

4. The oil content and fatty acid composition of the maize kernels was extremely stable, being only influenced to a slight extent by nutrient supplies and the year. During the experimental period excessive N supplies only reduced the oil content and modified the fatty acid composition on one occasion. There was no substantial change in the oil content or fatty acid composition as a function of P supplies.

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# EFFECTS OF ACID TREATMENT AND COMPOSTING ON BONES USED AS FERTILIZER

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The aim of the work was to analyse the compostable properties of bone powder produced via different treatment methods and industrial conditions, and to study their effect on plant growth and phosphorus uptake. The bones were treated in water with different temperatures, bone-water ratios and treatment times. Further treatment was carried out with citric, nitric and sulphuric acid with different concentrations, temperatures, bone-water ratios and treatment times. Industrial bone powder was composted under model industrial conditions.

The available phosphorus content of these materials was estimated using ryegrass (*Lolium perenne*) as indicator plant in a climatic chamber.

The water-soluble phosphorus content of the bones increased in the citric acid and sulphuric acid treatment, depending on the water treatment conditions and the acid concentration. This increase amounted to about 30 times (0.32–8.51 mg/100 mg) compared to the water treatment.

The results of the plant test demonstrated that the phophorus content of treated bone powder and compost was readily available to plants. The phosphorus content of the compost was available over a longer period.

**Key words**: bone powder, acid treatment, water-soluble phosphate, compost, phosphate availability

## Introduction

Bones are not suitable as fertilizers in their original condition because they are almost insoluble in soil and are thus unavailable to plants. The mineral part of bones consists mainly of phosphorus and calcium compounds: tri-calcium phosphate (84%), calcium carbonate (10%), calcium citrate (2%), di-sodium hydrogen phosphate (2%) and hydroxyapatite and fluoroapatite (2%). In addition, numerous biologically essential microelements can be found in bones (Zn, Cu, Mn, Fe).

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Bone ash contains about 36% Ca and 17% P, giving a Ca/P ratio of approximately 2:1. Previously guano or Thomas-cinder, a by-product of steel production, were used as phosphorus fertilizers. These products did not have to be modified chemically to make them suitable as fertilizers. Bone powder was created by chopping and boiling bones, extracting the fat and glue from it, and then crushing it to a powder. Bone powder is a very good phosphorus fertilizer and also ameliorates acidic soils. It has been used in China for hundreds of years. In the early 19$^{th}$ century there was increased interest in bones in England, and bone imports from the continent reached 30 thousand tons a year (Cserháti and Kosutány, 1887; Kádár, 1995).

In his famous study on agricultural chemistry Liebig (1840) detailed the importance of phosphorus in the organic material production of plants and suggested treating poorly soluble phosphates with sulphuric acid to produce an easily soluble form.

In England, independently from the work of Liebig, Sir John Bennet Lawes patented super-phosphate manufactured from bones via acid treatment in 1842 and started industrial production of the fertilizer in the same year. Due to the intensive production, the bone powder available as raw material was insufficient. Liebig modified his patent in 1848 to enable super-phosphate to be produced from mineral apatite and phosphorite, but super-phosphate produced from bones was more valuable from the biological point of view (Kádár, 1995). Following the successful use of bone treatment in England and Holland, research was carried out in the Lower Austria region of the Austro-Hungarian Monarchy on wheat, sugar beet and potato plants using imported bone powder. The conclusion was that bone powder alone increased production, but by applying nitrogen-containing materials (ammonium salts, oilcake) or by growing forecrops which left significant amounts of nitrogen in the soil even better yields could be achieved (Anonymous, 1856). Extraction with mineral acids (e.g. hydrochloric acid, sulphuric acid, nitric acid) improves the water-soluble phosphorus content of bones, making it available to the roots. During the production of superphosphate, fluorapatite and hydroxyapatite are converted to water-soluble calcium phosphate due to the effect of sulphuric acid.

Later, particularly from the 1950s onwards, improved extraction technologies allowed bones to be used as additives in animal feeding. Since the 1980s bone protein products have also been used in the production of food, medication, sanitary products, cosmetics and animal feed (Hegedűs et al., 1998).

Due to the outbreak of Mad Cow Disease in Western Europe in 2001 stricter food processing regulations were implemented. Since September 2003 proteins of animal origin cannot be fed to animals bred for their meat.

Nowadays bone powder is used as a fertilizer for various crops, particularly for horticultural crops grown in soil or other media. The most important property of this material is the availability of phosphorus and calcium after extraction (Faria et al., 2001).

Natural untreated bone powder is commonly used in ecological farming systems.

Approximately ten thousand tons of bone scrap are produced annually by the Hungarian meat industry. Attempts are thus being made to develop a modern, environment-friendly bone extraction method and to use the product in environment-friendly agriculture.

The aim of this work was to increase the water-soluble phosphorus content of the bones, to examine the feasibility of composting bone powder and to test the effect of bone treatment technologies on plant growth and phosphorus uptake.

## Materials and methods

*Extraction*

The extractions were carried out in the Central Food Science Research Institute (KÉKI, 2004) in pressure- and acid-proof bins to which a device was attached to register temperature and pressure.

The laboratory extraction of commercial pig bones was carried out at 135°C and at a pressure of 3.192 bar. The bones were kept at the specified temperature for 60 min in the case of water extraction and 30 minutes in all other cases, with a bone to water ratio of 1:2 (Table 1).

The raw material of the 10% citric acid and 2 N sulphuric acid treatments was commercial pig bones boiled at 135°C for 60 min. After boiling the pig bones were treated for 30 minutes at 135°C with citric acid or 2 N (9.1%) sulphuric acid. The bone to acid ratio was 2:1.

Following the acid extractions the bones were rinsed with water, dried for 16 h at 80°C and ground in a laboratory chopper, after which the water-soluble phosphorus content was determined. A similar method was used for commercial chicken bones, except that the temperature was 145°C and the concentration of citric acid was 20 %, while that of sulphuric acid was 1 N.

Pig bones from the slaughterhouse were treated under industrial conditions in the bone-processing plant of the Bakonyalja Corporation in Bakonykoppány. The bones were boiled for 90 min at 130°C (4 bar) in containers to which steam was gradually added, then dried and ground. The industrial bone powder was treated with 70% sulphuric acid or 65% nitric acid at 55°C for 30 min (Table 1).

*Table 1*
Laboratory conditions for bone treatment (Budapest, 2003)

| Sample code | Temperature, °C | Duration, min | Extracting solution (Bone:water ratio 1:2) |
|---|---|---|---|
| C. I/1 | 145 | 20 | Water |
| C. I/2 | 145 | 40 | 20% citric acid |
| C. I/3 | 145 | 20 | 1 N sulphuric acid |
| P. I/4 | 135 | 60 | Water |
| P. I./5 | 135 | 30 | 2 N sulphuric acid |
| P. I/6 | 135 | 30 | 10% citric acid |
| I. I/7 | ≈55 | 30 | 70% sulphuric acid |
| I. I/8 | ≈55 | 30 | 65% nitric acid |

C: Commercial chicken breast-bones; P: Commercial pig bones; I: Industrial bone powder or pig bones from the slaughterhouse

*Nitric acid extraction and neutralization*

Commercial pig bones boiled in water at 135°C for 60 min, then ground, were used as raw material. The nitric acid extraction was carried out in the laboratory in an open glass container without the application of heat. When 50 ml of 65% nitric acid was added to 100 g boiled, ground pork bones, the temperature rose to 55–65°C. Quantities of 20, 25, 30 or 35 ml of 60% potassium hydroxide were added to neutralise the acidity (Table 2). The resulting material was dried for 16 h at 80°C, then chopped in the laboratory chopper, after which the water-soluble phosphorus content and pH were determined.

In the case of industrial bone powder, 100 g bone powder was wetted with 30 ml water, then 50 ml of 65% nitric acid was added, after which the acidity of the solution was reduced with 10–60 ml of 60% potassium hydroxide (Table 3). The resulting bone powder was dried for 16 h at 80°C, then chopped in the laboratory chopper, after which the water-soluble phosphorus content and pH were determined.

*Composting under semi-industrial conditions*

Compost I: 6 $m^3$ chicken manure + straw, 300 kg bone powder, 100 l water

Compost II: 6 $m^3$ chicken manure + straw, 800 l technological bone liquid

Compost III: 6 $m^3$ chicken manure + straw, 300 kg bone powder, 1200 l technological bone liquid

The bone liquid was gained as a by-product of bone powder production.

The components were mixed in a fertilizer spreader, while water and technological bone liquid were sprayed on the substrate. Two triangular heaps, 4 × 1.5 m at the base, 1.5 m high, with a volume of 3 $m^3$, were formed in a covered place for each treatment.

During the composting process the product was turned over weekly, and the temperature was recorded every day.

The results of chemical analysis on the matured composts can be found in Table 4.

*Laboratory methods*

The laboratory measurements were carried out at the Department of Soil Science and Agrochemistry of Szent István University (SZIE-TALT, 2004).

The moisture content of the composts was determined by drying the samples in an oven at 105°C to constant weight (Kehres, 1998a). The total organic matter content was calculated from the ignition loss (at 600°C for 5 hours; Kehres, 1998b). The pH was measured by the direct potentiometric method. After calibration a suspension was made using 2.5 g sample and 12.5 ml 1 M KCl (Kehres, 1998c). The total nitrogen, phosphorus and potassium contents of the samples were determined after sulphuric acid+hydrogen peroxide digestion. Nitrogen was determined by Parnass-Wagner steam distillation (Kehres, 1998d), total phosphorus colorimetrically, and potassium by flame photometry (Kehres, 1998e; Sarkadi et al., 1955)

The easily soluble nitrogenous compounds in the samples were determined after 1% KCl extraction in the Parnass-Wagner device. The C/N ratio was calculated from the total nitrogen content and organic matter content using the formula: organic matter content (dry matter) / 1.725 × nitrogen content (dry matter) (VKS, 2001).

*Table 2*

Boiled pig bones treated with nitric acid and neutralized (Budapest, 2003)

| Sample code | Pre-treatment | Treatment |
|---|---|---|
| P. II/1 | Boiling at 135°C for 60 min, grinding | 100 g pre-treated bone powder + 50 ml 65% $HNO_3$ |
| P. II/2 | P. II/1 treatment + extraction with 65% $HNO_3$ | P. II/1 treatment + 20 ml 60% KOH |
| P. II/3 | P. II/1 treatment + extraction with 65% $HNO_3$ | P. II/1 treatment + 25 ml 60% KOH |
| P. II/4. | P. II/1 treatment + extraction with 65% $HNO_3$ | P. II/1 treatment + 30 ml 60% KOH |
| P. II/5. | P. II/1 treatment + extraction with 65% $HNO_3$ | P. II/1 treatment + 35 ml 60% KOH |

P: Commercial pig bones

*Table 3*
Industrial bone powder (I.) treated with nitric acid and neutralized (Budapest, 2003)

| Extraction code | Pre-treatment | Treatment |
|---|---|---|
| I. II/1 | Boiling at 130°C for 90 min, grinding | 100 g bone powder + 30 ml water + 50 ml 65% nitric acid |
| I. II/2 | I. II/1 treatment + extraction with 65% $HNO_3$ | Extraction I. II/1 + 10 ml 60% KOH |
| I. II/3 | I. II/1 treatment + extraction with 65% $HNO_3$ | Extraction I. II/1 + 20 ml 60% KOH |
| I. II/4 | I. II/1 treatment + extraction with 65% $HNO_3$ | Extraction I. II/1 + 30 ml 60% KOH |
| I. II/5 | I. II/1 treatment + extraction with 65% $HNO_3$ | Extraction I. II/1 + 40 ml 60% KOH |
| I. II/6 | I. II/1 treatment + extraction with 65% $HNO_3$ | Extraction I. II/1 + 50 ml 60% KOH |
| I. II/7 | I. II/1 treatment + extraction with 65% $HNO_3$ | Extraction I. II/1 + 60 ml 60% KOH |

*Table 4*
Properties of samples taken on the 178th day of composting (Gödöllő, 2003)

| Sample code | Dry matter % | Organic matter % | C/N ratio | $pH_{KCl}$ | $NH_4$-N mg/100g | $NO_3$-N mg/100g | N% | $P_2O_5$ % | $K_2O$% |
|---|---|---|---|---|---|---|---|---|---|
| Compost I | 75.03 | 55.03 | 14.5 | 8.32 | 220.91 | 0 | 2.20 | 5.27 | 3.04 |
| Compost II | 73.22 | 27.73 | 7.6 | 8.81 | 123.46 | 12.77 | 2.12 | 4.41 | 2.37 |
| Compost III | 70.44 | 38.08 | 7.05 | 8.42 | 140.91 | 2.39 | 3.13 | 7.58 | 4.38 |
| $LSD_{5\%}$ | 2.31 | 13.78 | 4.15 | 0.26 | 51.96 | 6.79 | 0.56 | 1.64 | 1.02 |

*Pot experiment*

The pot experiments were carried out at the Department of Soil Science and Agrochemistry, Szent István University.

Each 1-litre pot contained 700 g of low fertility soil (brown forest soil from Gödöllő), and identical amounts (100 mg/kg) of $P_2O_5$ from bone powder treated in various ways were mixed with each sample. The properties of the soil can be seen in Table 5. The $P_2O_5$ amount was determined based on the phosphorus content of the samples. Urea and 40% KCl fertilizer were added to the pots so as to provide equal amounts of nitrogen and potassium based on the properties of the soil and the demands of the crop. The amounts can be seen in Table 6.

Ryegrass (*Lolium perenne*) was used as test plant, with 1 g seed in each pot. Each treatment was carried out in three replications. The pots were placed in a climate chamber at a temperature of 20°C, moisture content 60%, light intensity 6000 lux, and daily light period 12 hours. The pots were sprayed with distilled water every day. The plants were first cut 18 days after emergence, then on day 35. The fresh weight of the plants was measured following cutting, then the weight of dry matter was measured after drying. The phosphorus content of the plants was determined after sulphuric acid+hydrogen peroxide digestion. Table 5 contains information on the soil used in the experiment, and Table 6 on the element content of the materials used in the experiment.

*Table 5*
Properties of soil used in the pot experiment (Gödöllő, 2003)

| Parameter | Value |
|---|---|
| Texture, $K_A$ | 32 |
| $pH_{KCl}$ | 6.3 |
| $pH_{H2O}$ | 7.07 |
| Humus content, % | 2.39 |
| $CaCO_3$ | 0 |
| AL-$P_2O_5$ ppm | 29.2 |
| AL-$K_2O$ ppm | 80 |

*Table 6*
Element content of materials used in the experiment (Gödöllő, 2003)

| Raw material | "Total" element content, % | | | N added as urea, g/700 g soil | K added as 40% KCl, g/700 g soil |
|---|---|---|---|---|---|
| | P | K | N | | |
| Control (low fertility soil) | 0.0037 | 0.0090 | 0.065 | – | – |
| Compost (trade) | 0.67 | 0.92 | 1.42 | – | – |
| Compost I | 2.32 | 2.52 | 2.20 | 0.112 | 0.075 |
| Compost II | 1.94 | 1.97 | 2.12 | 0.101 | 0.08 |
| Compost III | 3.34 | 3.64 | 3.13 | 0.112 | 0.075 |
| Bone powder (pig bone, industrial extraction) | 4.80 | 0.44 | 3.12 | 0.131 | 0.167 |
| C. I/1. | 3.50 | 1.54 | 9.16 | 0 | 0.135 |
| C. I/2. | 7.00 | 0.27 | 3.69 | 0.139 | 0.171 |
| C. I/3. | 6.00 | 5.11 | 2.42 | 0.148 | 0.098 |
| P. I/4. | 12.00 | 0.64 | 2.93 | 0.158 | 0.170 |
| P. I/5. | 13.00 | 1.44 | 1.27 | 0.167 | 0.165 |
| P. I/6. | 14.00 | 0.49 | 3.18 | 0.159 | 0.172 |
| I. I/7. | 12.17 | 1.10 | 2.54 | 0.569 | 0.270 |
| I. I/8. | 7.33 | 2.87 | 2.62 | 0.0853 | 0.248 |
| $LSD_{5\%}$ extractions+bone powder | 3.93 | 1.56 | 2.250 | – | – |
| $LSD_{5\%}$ total | 4.79 | 1.46 | 2.05 | – | – |

## Results and discussion

Tests were made on the phosphorus content of bone powder originating from different animals (pig, chicken) and extracted using different methods (extractant, temperature, duration, etc.), and changes in pH occurring as a result of the treatment were recorded. In addition to chemical extraction, bone powder produced under industrial conditions was composted after mixing with various raw materials.

The water-soluble phosphorus content of boiled chicken and pig bones treated with citric acid and sulphuric acid significantly increased compared to that of bones treated only with water (Table 7). The highest water-soluble phosphorus content was found in chicken bones treated with 20% citric acid (2.95 mg P 100 $g^{-1}$). The water-soluble phosphorus content of bone powder made from pig bones from the slaughterhouse, pre-treated at high pressure and temperature under industrial conditions, followed by sulphuric acid and nitric acid treatment in the laboratory, was 8–11 mg P 100 $g^{-1}$.

The nitric acid extraction of boiled pig bones (Table 8) resulted in an increase in the phosphorus content to 8.51 mg 100 $g^{-1}$, but the water-soluble phosphorus content decreased to 2.46 mg 100 $g^{-1}$ after neutralization with KOH, while the pH increased from 3.56 to 4.60.

Industrial bone powder treated with nitric acid without neutralization (Table 9) had a water-soluble phosphorus content of 6.49 mg 100 $g^{-1}$, the water-soluble phosphorus content decreasing to 1.27 mg 100 $g^{-1}$ after maximum KOH neutralization, while the pH increased from 3.58 to 5.84.

*Table 7*
Water-soluble phosphorus content (mg P/100 mg bone powder ) of bones after acid treatment (2003)

| Experiment code | Treatment | Water-soluble phosphorus content |
|---|---|---|
| C. I/1 | 145°C, 20 min, water | 0.31±0.01 |
| C. I/2 | 145°C, 40 min, 20% citric acid | 2.95±0.07 |
| C. I/3 | 145°C, 20 min, 1 N sulphuric acid | 2.38±0.13 |
| P. I/4 | 135°C, 60 min, water | 0.32±0.02 |
| P. I/5 | 135°C, 30 min, 2 N sulphuric acid | 2.01±0.14 |
| P. I/6 | 135°C, 30 min, 10% citric acid | 0.96±0.09 |
| I. I/7 | 55°C, 30 min, 70% sulphuric acid | 10.95±0.29 |
| I. I/8 | 55°C, 30 min, 65% nitric acid | 8.39±0.17 |
| $LSD_{5\%}$ | | 0.20 |

C: Commercial chicken breast-bones; P: Commercial pig bones; I: Industrial bone powder, pork bones from the slaughterhouse

*Table 8*
Water-soluble phosphorus content (mg P/100 mg bone powder) and pH measured after nitric acid treatment and potassium hydroxide neutralization of boiled commercial pig bones (for details, see Table 2; Budapest, 2003)

| Experiment code | Treatment | Water-soluble phosphorus content | pH |
|---|---|---|---|
| P. II/1 | A | 8.51 ± 0.22 | 3.56 |
| P. II/2 | B | 3.93 ± 0.14 | 3.93 |
| P. II/3 | B | 2.77 ± 0.06 | 4.24 |
| P. II/4 | B | 2.60 ± 0.10 | 4.32 |
| P. II/5 | B | 2.46 ± 0.10 | 4.60 |
| Super-phosphate | | 8.04 ± 0.59 | 3.58 |
| $LSD_{5\%}$ | | 0.382 | 0.42 |

A: Boiling at 135°C for 60 min, grinding; B: Boiling at 135°C for 60 min, grinding, extraction with 65% $HNO_3$

*Table 9*
Water-soluble phosphorus content (mg P/100 mg bone powder) and pH measured after nitric acid treatment and potassium hydroxide neutralization of industrial pig bones (for details, see Table 3) (Budapest, 2003)

| Experiment code | Treatment | Water-soluble phosphorus content | pH |
|---|---|---|---|
| I. II/1 | A | 6.49 ± 0.32 | 3.58 |
| I. II/2 | B | 4.56 ± 0.21 | 3.90 |
| I. II/3 | C | 4.42 ± 0.13 | 4.12 |
| I. II/4 | C | 1.67 ± 0.06 | 4.75 |
| I. II/5 | C | 1.35 ± 0.03 | 5.06 |
| I. II/6 | C | 1.27 ± 0.21 | 5.26 |
| I. II/7 | C | 0.94 ± 0.02 | 5.84 |
| $LSD_{5\%}$ | | 0.25 | 0.81 |

A: Boiling at 130°C for 90 min, grinding; B Boiling at 130°C for 90 minutes, grinding, extraction with 65% $HNO_3$; C: Boiling at 130°C for 90 min, grinding, extraction with 65% $HNO_3$

The mobilization of the phosphorus found in bone powder was observed during the composting process, and the available phosphorus content from different compost materials and bone powders was examined in a pot experiment with two cuts of ryegrass.

The fresh mass of the first cut of ryegrass was much the same in all the treatments, with the exception of treatments 7 and 8, where it was significantly smaller. This is probably due to the close C/N ratio, and/or the result of gases created during the biological degradation of the organic matter in the bone powder, which cause growth depression. Another reason for this phenomenon could have been the slow mobility of the nutrients in industrial bone powder. In the second cut there was a significantly higher amount of fresh mass produced in the Compost II treatment and significantly less in treatments 5 and 7. As in the case of fresh mass, the quantity of dry matter was similar in all the treatments in the first cut, with the exception of treatments 7 and 8, where it was much smaller, probably for the same reasons. The dry matter yield of the second cut was generally higher in the compost treatments than in the case of extracted bone powders. The yield was substantially smaller in treatment 5, where the nitrogen content of the extracted bones was the smallest and urea was given as nitrogen additive, which could cause growth depression. The yield was also low in extractions 7 and 8, which can be explained by the reasons given above. Based on the dry matter yield the different fertilizers (composts and bone powders extracted in different ways) had similar effects on ryegrass production. Only pig bones treated with a low concentration of sulphuric acid or a high concentration of sulphuric and nitric acids showed different results.

The percentage phosphorus content of ryegrass was also similar in the first cut of ryegrass plants in different treatments (0.32–0.47%, $LSD_{5\%}$ 0.06). The phosphorus concentration was the highest in plants in treatment 8, probably due to their low mass. The lowest phosphorus concentration was measured in the case of treatment 1. By contrast, in the second cut the phosphorus content decreased a little in most treatments (to approximately 0.22%). The phosphorus concentration of the low-yielding plants in treatments 7 and 8 increased further to 0.6%. The phosphorus uptake was the highest in treatment 3, probably due to the loosely bound form of phosphorus in young chicken bones, which is readily soluble in sulphuric acid. The lowest phosphorus uptake was measured in treatment 7. The phosphorus uptake was smaller in the second cut. Plants grown in compost-treated soils utilized more phosphorus than plants treated with extracted bone powder, because the phosphorus fixed in the organic matter can only be taken up following mineralization, which was only just starting at the time of the second cut.

Summarizing the results of the pot experiment it can be concluded that phosphorus was taken up by ryegrass similarly from bone powders treated in different ways and when added to the soil in compost. The only difference was that the phosphorus content of compost was available to the plants for a longer

period, probably due to slower uptake. There was no significant difference between the bone treatment methods from the point of view of plant phosphorus uptake, except when the bone powder was treated with sulphuric acid (treatment 8), where the results were somewhat lower.

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*Short communication*

# CORRELATION ANALYSIS OF YIELDS AND RELATED PHYSIOLOGICAL VARIABLES IN TWELVE GENERATIONS OF DURUM WHEAT

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The experimental material comprised three crosses, namely Cocorit 71 × A-9-30-1, HI 8062 × JNK-4W-128 and Raj 911 × DWL 5002, generated from six diverse parents. Twelve populations of each of these three crosses revealed that sufficient genetic variation was recorded among generations for all the traits in three crosses. The results of a correlation study demonstrated that the grain yield per plant was significantly and positively associated with peduncle area and flag leaf area in the cross Cocorit 71 × A-9-30-1. However, the spike area had poor correlation with grain yield. In the cross HI 8062 × JNK-4W-128, grain yield per plant was positively associated with peduncle area and spike area, whereas flag leaf area showed a positive but weak association with grain yield. In the cross Raj 911 × DWL 5002, grain yield per plant was positively associated with all three physiological traits studied, indicating that improvement in grain yield may be made by these traits in this material. Peduncle area appeared to be the most important trait in the present study because of its association with grain yield in all three crosses. Although flag leaf area and spike area showed a positive association with grain yield in all three crosses, their relative magnitudes and significance changed from one cross to the other. Correlation studies revealed that selection for peduncle area would lead to high yield in durum wheat. However, due consideration should also be given to flag leaf area and spike area during the selection of plants for further tangible advances in grain yield in durum owing to their positive association with grain yield.

**Key words:** durum wheat, genetic variation, physiological traits, grain yield, correlation

## Introduction

India has 2.5 million hectares of durum wheat (*Triticum durum*), which is widely cultivated in many parts of central and peninsular India. The interest and attention of agronomists, scientists and economists in durum has increased in recent decades, and it is becoming popular under high fertility irrigated conditions in North India. It is the second most important wheat species in the

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country, so any further improvement in the species would be helpful in meeting the food requirements of the burgeoning population of India. Grain yield is the end-product of interactions between many factors, known as yield-contributing components, and is a complex trait. Selection based on grain yield is rarely useful, but that based on its component characters could be more effective. Basic information on major physiological yield-contributing characters is essential to ensure efficient selection for higher yield. In the case of aestivum wheat, a number of findings have been reported based on fixed genotypes and segregating populations, but there is very little information on durum wheat from correlation studies, particularly in segregating populations. Therefore, the present study was conducted to collect information on the association of three physiological traits with grain yield in twelve generations of durum wheat.

## Materials and methods

The experimental material comprised three crosses, namely Cocorit 71 × A-9-30-1, HI 8062 × JNK-4W-128 and Raj 911 × DWL 5002, generated from six diverse parents. Twelve basic generations, namely two parents, $F_1$ and $F_2$, first backcross generations with both parents ($BC_1$ and $BC_2$), where $BC_1$ was the cross between $F_1$ × female parent and $BC_2$ was $F_1$ × male parent, their selfed progenies ($BC_1$ $F_2$, $BC_2$ $F_2$) and the second backcross generations ($BC_{11}$, $BC_{12}$, $BC_{21}$ and $BC_{22}$), i.e. the $BC_1$ and $BC_2$ plants crossed with both original parents ($BC_1$ × female parent; $BC_1$ × male parent, and $BC_2$ × female parent; $BC_2$ × male parent). These twelve populations of each of the three crosses were evaluated in a randomized block design with three replications. Each replicate was divided into three compact blocks. The crosses, each consisting of twelve populations, were randomly allotted to the blocks. All twelve generations were then randomly allotted to twelve plots within a block. The plots of various generations contained different numbers of rows, i.e. each parent and $F_1$ plot consisted of two rows, while each backcross generation was sown in four rows, and the $F_2$ and the second cycle of backcrosses in six rows. Each row was 5 m long, accommodating 33 plants spaced 15 cm apart, the row to row distance being 30 cm. Border rows were provided at the beginning and end of the experimental rows in each block. The experiment was planted at the Research Farm of Rajasthan Agriculture University, Agricultural Research Station, Durgapura, Jaipur, Rajasthan, India in the 'rabi' crop season ($42^{nd}$ week). Observations on peduncle area ($cm^2$), flag leaf area ($cm^2$), spike area ($cm^2$) and grain yield per plant were recorded on 15 random plants in each parent and $F_1$, 30 plants in each backcross generation and 60 plants in each $F_2$ and second backcross generation in each replication. Peduncle area and spike area were calculated using the method suggested by Yap and Harvey (1972). Flag leaf area was calculated with the method outlined by Simpson (1968). The data were analysed statistically with the method of Panse and Sukhatme (1967) and the correlation coefficient was calculated following the method of Dewey and Lu (1959).

## Results and discussion

In the analysis of variance the mean squares due to generations were found to be highly significant for all four characters studied in three crosses of durum wheat, indicating sufficient genetic variation among the generations for all the traits in the three crosses involved in the present study.

In order to identify physiological plant traits suitable for selection for the further amelioration of grain yield in durum wheat, the correlation coefficients among various physiological characters were calculated in twelve generations of three crosses (Table 1). The results of the correlation study revealed that the grain yield per plant was significantly and positively associated with peduncle area and flag leaf area in the cross Cocorit 71 × A-9-30-1. However, spike area had a poor correlation with grain yield, which may be attributed either to high sampling error or limited variations in the generations in this cross. In the cross HI 8062 × JNK-4W-128, the grain yield per plant was positively associated with peduncle area and spike area, whereas the flag leaf area showed a positive but weak association with grain yield. Although flag leaf area has considerable heritability, the limited variation in the progenies may restrict its exploitation through selection in this material. In the cross Raj 911 × DWL 5002, grain yield per plant was positively associated with all three physiological traits studied, indicating that improvement in grain yield may be based on these traits in this material. Earlier studies (Kaltsikes and Lee, 1971; Sidhu et al., 1976; De Pace et al., 1978; Thakral et al., 1979; Sharma and Singh, 1983; Kumar and Chowdhury, 1986; Srivastava et al., 1993; Dhanda and Sethi, 1996) substantiate this point.

Experimentally it is evident that direct selection for grain yield cannot be practised on the basis of correlations, especially under normal sowing conditions, because its low heritability, and the involvement of a large amount of non-additive variation within and between environments could limit progress through selection (Blum, 1988; Acevedo et al., 1991). Therefore, selection for characters having high heritability and relatively simple inheritance could be more fruitful than that for grain yield alone. Peduncle area appeared to be the most important trait in the present study because of its association with grain yield in all three crosses. Flag leaf area and spike area, though they showed a positive association with grain yield in all three crosses, exhibited changes in relative magnitude and significance in different crosses. Although these traits have considerable heritability, the limited variation between the generations may restrict exploitation through selection in a given material.

*Table 1*

Correlation coefficients between physiological traits and grain yield in three crosses of durum wheat

| Cross | Character | Correlation coefficient |
|---|---|---|
| Cocorit 71 × A-9-30-1 | Peduncle area | 0.5861* |
| | Flag leaf area | 0.8205** |
| | Spike area | 0.4013 |
| HI 8062 × JNK-4W-128 | Peduncle area | 0.9759** |
| | Flag leaf area | 0.3427 |
| | Spike area | 0.7852** |
| Raj 911 × DWL 5002 | Peduncle area | 0.7324** |
| | Flag leaf area | 0.6591* |
| | Spike area | 0.6572* |

* and **: significant at the 5% and 1% level of probability, respectively

Spike area and flag leaf area showed a poor correlation with grain yield per plant in the crosses Cocorit 71 × A-9-30-1 and HI 8062 × JNK-4W-128, respectively, but in other cases both traits showed a positive significant association with grain yield per plant, indicating that the role of physiological trait(s) changed depending on the material studied. Choudhary et al. (1985), Sharma and Kaul (1986),Verma and Yunus (1993) and Srivastava et al. (1993) also reported that the grain yield was strongly dependent on one or more physiological traits. Thus, the correlation studies indicated that peduncle area was the most important trait for improving the grain yield in durums. However, the flag leaf area and spike area also appeared to be important characters owing to their positive association with grain yield in all the crosses studied.

In conclusion, correlation studies revealed that selection for peduncle area would lead to high yield in durum wheat. However, due consideration should also be given to flag leaf area and spike area during the selection of plants for a further tangible advance in grain yield in durums owing to the positive association with grain yield. Many wheat workers studied the gene action involved in these physiological traits (Jain and Singh, 1976; Ilychenko, 1977; Bariga, 1979; Dhindsa, 1982; Prabhu and Sharma, 1984; Srivastava et al., 1992; Singh, 2002) and indicated that these characters were predominantly governed by additive genetic variances with the involvement of dominance and epistatic effects. Therefore, selection would be effective if dominance and epistatic effects were reduced after a few generations of selfing and/or intermating in early segregating generations (Singh et al., 1986). This would not only reassemble the adaptive genes in the population but also increase the population mean and retain greater variability for selection over a longer span of time.

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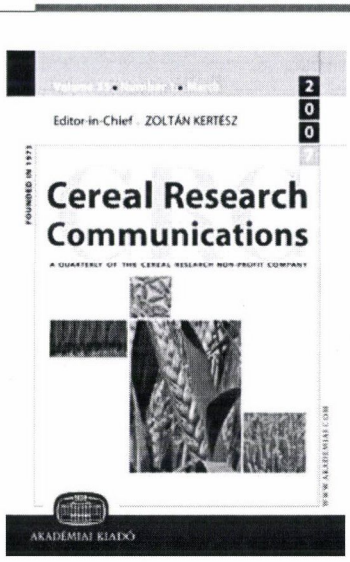
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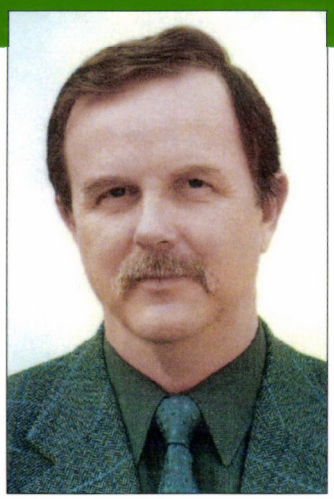
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# CONTENTS

ORIGINAL PAPERS

Electrophoretic and immunochemical techniques for the molecular reassessment of relationships within *Vicieae*
*R. H. Sammour* ..... 131

Incorporation of leaf rust resistance genes into winter wheat genotypes using marker-assisted selection
*M. Gál, G. Vida, A. Uhrin, Z. Bedő* and *O. Veisz* ..... 149

Isolated microspore cultures of a Hungarian durum wheat (*Triticum turgidum* L.) cultivar, Martondur 1
*F. Bakos, A. Fábián* and *B. Barnabás* ..... 157

Kinetics of cadmium in different Indian and Hungarian soils: Incubation study at field capacity
*B. S. Panwar, M. S. Grewal* and *L. Márton* ..... 165

Effects of nitrogen fertilisation and two growing sites on biomass production and lignan content of linseed (*Linum usitatissimum* L.): Second year
*R. Zimmermann, U. Bauermann* and *C. Spedding* ..... 173

Effects of *Glomus mosseae* strains of different origin on plant macro- and micronutrient uptake in Cd-polluted and unpolluted soils
*I. Biró* and *T. Takács* ..... 183

Effect of temperature and radiation on photosynthesis productivity in chestnut populations (*Castanea sativa* Mill. cv. Judia)
*P. Almeida, L.-T. Dinis, J. Coutinho, T. Pinto, R. Anjos, J. Ferreira-Cardoso, M. Pimentel-Pereira, F. Peixoto* and *J. Gomes-Laranjo* ..... 193

Effect of integrated nutrient management in a soybean–wheat crop sequence on the yield, micronutrient uptake and post-harvest availability of micronutrients on Typic Ustochrepts soil
*S. K. Dadhich* and *L. L. Somani* ..... 205

Chemical quality parameters of maize hybrids in various FAO maturity groups as correlated with yield and yield components
*Z. Hegyi, I. Pók, C. Szőke* and *J. Pintér* ..... 217

Yield of switchgrass *(Panicum virgatum* L.*)* as influenced by cutting management
*Z. Kiss, A. F. Fieldsend* and *D. D. Wolf* ..... 227

Rheological properties of wheat dough in some hexaploid Indian wheat varieties
*K. Gajalakshmi* and *V. R. K. Reddy* ..... 235

Importance of plant growth-promoting rhizobacteria in enhancing the seed germination and growth of watermelon attacked by fungal pathogens
*S. Lokesh, B. G. Bharath, V. B. Raghavendra* and *M. Govindappa* ..... 243

Effect of different sowing densities on some characteristics of *Isatis tinctoria* L. and *Isatis constricta* Davis and on the recovery of indican
*S. Kizil, N. Arslan* and *K. M. Khawar* ..... 251

SHORT COMMUNICATION

Estimates of variation and heritability of some quantitative and quality characters in *Triticum turgidum* L. ssp. *durum* (Desf.)
*S. N. Sharma* and *Y. Sharma* ..... 261

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# ELECTROPHORETIC AND IMMUNOCHEMICAL TECHNIQUES FOR THE MOLECULAR REASSESSMENT OF RELATIONSHIPS WITHIN *VICIEAE*

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In this study, an array of electrophoretic and immunochemical techniques was used to investigate the legumins, vicilins and albumins of seed storage proteins in *Pisum sativum*, *Vicia faba*, *Lens esculentum*, and *Cicer arietinum* to delimit the boundary of the tribe *Vicieae* and to clarify the systematic position of the genus *Cicer*. The band patterns of the legumins of these species were broadly similar in that they had bands at Mr 60 kDa which disappeared in the presence of 2-mercaptoethanol, giving rise to two sets of new bands, at Mr approximately 40 kDa and 20 kDa, representing acidic or α and basic or ß subunits. The band patterns of the vicilins were also quite similar in that they showed bands at Mr approximately 71 kDa (convicilin) and 50 kDa (vicilin), which were not altered by the presence of 2-mercaptoethanol. Serologically, the legumins of *Vicia faba* and *Lens esculentum* exhibited total identity with *Pisum* legumin antiserum under non-reducing conditions, whereas the legumin of *Cicer arietinum* exhibited only partial identity, which was attributed to the failure of the low molecular subunit pair (Mr 33 kDa) to react with *Pisum* legumin antiserum. On the other hand, the vicilins of *Vicia faba*, *Lens esculentum* and *Cicer arietinum* had only partial identity with the vicilin of *Pisum sativum*, which was due to the failure of a number of subunits along the electrophoretic patterns of these species to react with *Pisum sativum* vicilin antiserum. The electrophoretic patterns of *Vicia faba*, *Lens esculentum* and *Cicer arietinum* were markedly different for the albumins. However, immunochemically they gave a positive reaction with *Pisum* major albumin antiserum (Mr 25 kDa) and showed a band with a molecular weight slightly higher than the major albumin of *Pisum sativum*. Extending the immunochemical study to members of the *Phaseoleae*, *Glycineae*, *Cajaneae* and *Diocleae* revealed that the vicilin and legumin of *Cicer* were more closely related to the *Vicieae* than to these tribes. Thus the data presented in this work recommended the classification of *Cicer* under *Vicieae* rather than as a separate tribe *Ciceridеae*.

**Key words**: tribe *Vicieae*, tribe *Ciceridеae*, legumins, vicilins, immunodiffusion, Western blotting

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## Introduction

*Vicieae* was first delineated as a "section", including *Aphaca*, *Cicer*, *Clymenum*, *Ervum*, *Lathyrus*, *Lens*, *Nissolia*, *Orobus*, *Pisum* and *Vicia*. Later on, it was given tribal rank by De Candolle (1825), who accepted *Cicer* L., *Ervum* L., *Faba* L., *Lathyrus* L., *Orobus* L., *Pisum* L. and *Vicia* L. as members of the tribe. However, the number of genera into which the species of *Vicieae* are grouped has tended to decrease with time. Bentham (1865) recognized six genera in the tribe *Vicieae*: *Cicer* L., *Vicia* L., *Lens* Miller, *Lathyrus* L., *Pisum* L. and *Abrus* L. The morphological, anatomical and karyological data advocated that *Abrus* should be excluded from the tribe *Vicieae* and placed in its own tribe *Abreae* (Streicher, 1902; Popov, 1928; Senn, 1938; Dormer, 1946; Hutchinson, 1967; Heywood, 1971).

The generic limits of the tribe *Vicieae* are still a matter of debate. This debate is essentially concentrated on the position of *Cicer* within the tribe. Reviewing anatomical, morphological, pollen grain morphology, karyological, isoflavonoid and isoenzymatic data, it was concluded that *Cicer* must be assigned to a separate tribe, the *Cicerideae* (Kupicha, 1977; Gapochka, 1984). Cladistic analysis based on morphological, anatomical, karyological and chemotaxonomical characteristics (Endo and Ohashi, 1997) and molecular phylogenies based on matK sequences (Wojciechowski et al., 2000) reached the same conclusion. On the other hand, immunological and electrophoretic data of the total seed proteins of members of the tribe *Vicieae* including *Cicer* displayed considerable homology, indicating that *Cicer* should be included in *Vicieae* (Kloz and Turkova, 1963; Simola, 1969; Tarlakovskaya, 1974; 1975; Gavriljuk, 1974; Cristofolini, 1981; Sammour, 1985). Furthermore, data on the DNA/DNA hybridization of 5-day-old seedlings of the members of the tribe *Vicieae* supported this conclusion (Sammour, 1991).

The controversy over the position of *Cicer* left the door open for further taxonomical studies on *Cicer* and its related species at the molecular level. Thus, this study aims to present information on the electrophoretic and immunological homology of the globulins (legumin, vicilin and convicilin) and the albumins of seed storage proteins in members of the tribe *Vicieae* with reference to the delimitation of the position of the genus *Cicer*.

## Materials and methods

*Materials*

Seeds of *Vicia faba* L. var. Giza 3, *Pisum sativum* var. Progress 9 and Victory, *Lens esculentum* var. Giza 9 and *Cicer arietinum* L. var. Partly 2, were obtained from the Agriculture Research Center, El Dokki, Egypt; seeds of jack bean (*Canavalia ensiformis*) were obtained as dry seeds from Sigma Chemical Co., Poole, Dorset, U.K.; seeds of *Glycine max*, *Phaseolus vulgaris*, *Phaseolus coccineus, Dolichos lablab, Cajanus cajan* and *Lathyrus odoratus* were obtained from the seed herbarium of Dr. P. Gates, Department of Biological Science, Durham University, U.K.; and seeds of *Vigna unguiculata* (L.) var. Walp were obtained from the International Institute of Tropical Agriculture (L. W. Lambourn & Co. Ltd., Croydon, U.K. All the chemicals used in the experiment were purchased from Sigma Chemical Co., Poole, Dorset, U.K. and were of reagent grade.

*Methods*

*Preparation of meal*

The seeds were freeze-dried for 30 min to remove surface moisture, then ground to a fine flour using a Janke and Kunkel water-cooled mill. The flour was passed through a 365 μm mesh Endicott sieve. Defatting was carried out by two hexane extractions (10 mL hexane/g meal) for 30 min at 0°C. After filtering off the hexane, the meal was air-dried under vacuum.

*Preparation of extracts*

Defatted meal from air-dried seeds was extracted with 50 mM sodium borate buffer pH 8.0 (10 mL buffer/g meal) at 4°C for 1 h, then centrifuged at 10.000 *g* for 20 min. The resulting precipitate was discarded and the supernatant was used for ammonium sulphate fractionation, gel electrophoresis or to prepare the globulin fraction.

*Ammonium sulphate preparation*

The total extract was fractionated by ammonium sulphate precipitation at 50, 70 and 90% relative saturation. The precipitates were collected by centrifugation at 10.000 *g*, then re-dissolved in 20 ml 0.05 M sodium borate buffer, pH 8.0. The supernatant at 90% relative saturation with ammonium sulphate and the re-dissolved precipitates were extensively dialysed against 5 mM sodium borate buffer, pH 8.0.

*Separation of the globulin fraction*

The total extract was extensively dialysed against 33 mM sodium acetate buffer, pH 4.7. The precipitated globulin proteins were separated from the albumin proteins still in solution by centrifugation at 12000 g. The precipitate was re-dissolved by suspending the pellet in water, then dialysed against 0.05 M sodium borate buffer, pH 8.0, prior to freeze-drying.

*Separation of the albumin fraction*

The total extract was extensively dialysed against 33 mM sodium acetate buffer, pH 4.7. The supernatant containing albumin was separated from the globulin by centrifugation at 12.000 *g*, extensively dialysed against distilled water and then freeze-dried.

*Purification of legumins*

The legumins were purified according to previously published procedures (Matta et al., 1981).

*Purification of vicilins*

The vicilins were purified according to previously published procedures (Croy et al., 1980).

*Immunodiffusion*

*Preparation of antiserums*

The *Pisum* vicillin antibodies and *Pisum* legumin antibodies used for immunodiffusion and the Western blotting analysis of *Vicieae* legumins and vicilins were prepared in rabbits according to the modified method of Hammer and Murphy (1993).

*Sample preparation*

Seed meal or purified proteins were dissolved in EDTA-Tris borate buffer [60.5 g Tris, 6.0 g EDTA (disodium salt) and 4.6 g $H_3BO_3$, in each L]. The buffer was adjusted to pH 4.0 by the addition of 6 N HCl.

*Gel preparation*

One g agarose was boiled for five minutes in 50 ml distilled water. Meanwhile, 50 ml Tris-EDTA-borate buffer, pH 9.6 was kept at 65°C. The boiled agarose was left at room temperature until its temperature dropped to 65°C and then mixed with the warm buffer. The mixture was cast in plastic plates. The plates were stored at 4°C for 2–4 hours and then holes were made, using a hexagonal array of outer wells around the central wells.

*Sample application*

Seed meal or purified samples were loaded in the outer wells and the antibody was loaded in the central well and left on a leveled table at 4°C for 24 hours (unless otherwise mentioned). The arcs formed after the incubation time were pressed, washed, stained with staining solution (0.0251 Coommassie Blue R250, 7% acetic acid, 50% methanol in water), and destained with destaining solution (7% acetic acid and 50% methanol in water).

*Western blotting technique*

After SDS-PAGE separation, the separated proteins were transferred to nitrocellulose paper by electro-blotting. The transferred proteins on nitrocellulose paper were immersed in specific antibodies, visualized by peroxidase-coupled antibodies and then stained with 4 chloro-1-naphthol (Towbin et al., 1979). The blotted proteins were separately detected using *Pisum* vicilin antibodies and *Pisum* legumin antibodies.

*Gel electrophoresis*

The seed meal was extracted with 0.125 M Tris/borate buffer, pH 8.9, containing 2% SDS and then analysed on 12% PAGE following the method of Laemmli (1970).

## Results

The seed globulins of *Vicia faba*, *Pisum sativum*, *Lens esculentum* and *Cicer arietinum* were examined using polyacrylamide gel electrophoresis, under reducing and non-reducing conditions (Fig. 1). The band patterns of all four species were broadly similar in that they showed bands at Mr approximately 71 kDa and 50 kDa, which were not altered by the presence of 2-mercaptoethanol; these bands are putatively due to convicilin and vicilin (Croy et al., 1980), respectively. The species also had bands at Mr 60 kDa under non-reducing conditions which disappeared in the presence of 2-mercaptoethanol, giving rise to two sets of new bands, at Mr approximately 40 kDa and Mr 20 kDa representing legumin, α and β subunits (Wright and Boulter, 1972; Croy et al., 1980; Gatehouse et al., 1981; Mendoza et al., 2001). Other minor bands showed much more marked species-to-species variation.

Total protein extracts of *Pisum sativum*, *Lens esculentum* and *Cicer arietinum* were analysed under non-reducing conditions in the first dimensions and under reducing conditions in the second dimensions (Fig. 2A–D). This identifies legumin subunits as "off the diagonal" disulphide subunit pairs (Matta et al., 1981). The second dimension patterns of different varieties of the same species were very similar in the number of subunits and the migration of the subunits, for instance, *Pisum sativum,* var. Victory and Progress 9 (Fig. 2A, B).

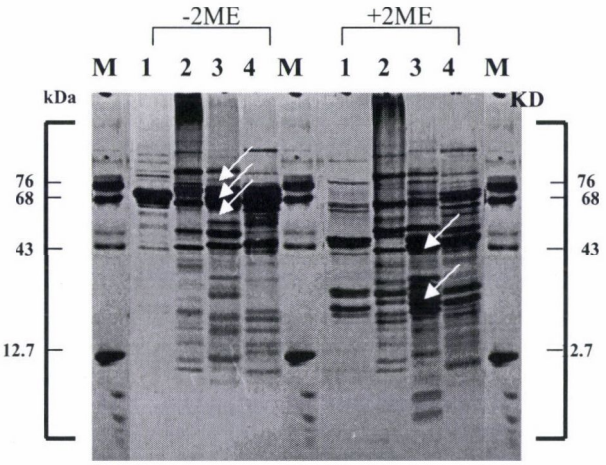



*Fig. 1.* SDS-PAGE of globulin seed proteins analysed under non-reducing and reducing conditions. 1, *V. faba*; 2, *P. sativum*; 3, *C. arietinum*; 4, *L. esculentum.* M: molecular weight-standard proteins. The arrows on the left point to bands with Mr of 71, 60 and 50 kDa; and those on the right to bands with Mr of 60 and 40 kDa

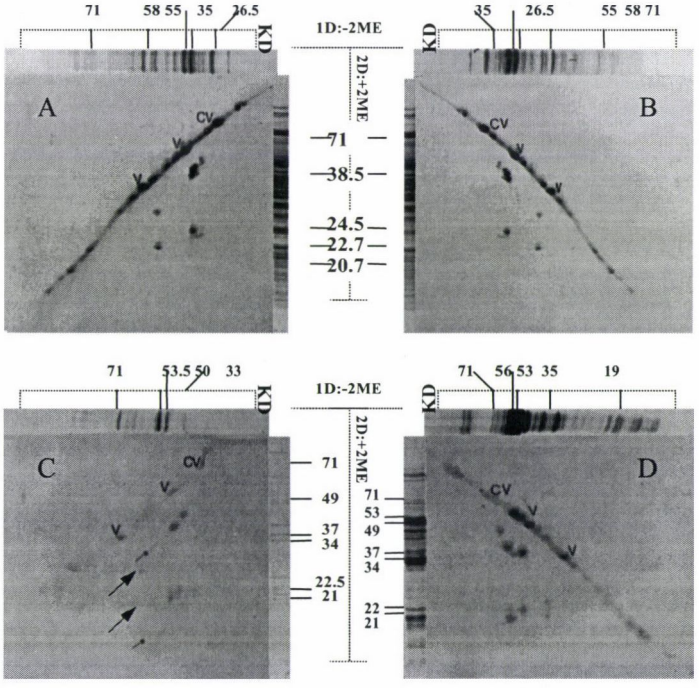



*Fig. 2.* Two-dimensional SDS-PAGE of total protein extracts. A, *P. sativum* var. Progress 9; B, *P. sativum* var. Victory; C, *C. arietinum;* D, *L. esculentum.* 1D: electrophoresis under non-reducing conditions in the first dimension. 2D: electrophoresis under reducing conditions in the second dimension

The second dimension patterns of *Pisum sativum, Lens esculentum* and *Cicer arietinum* showed the same number of subunit pairs (five) (Fig. 2A–D). All the former species were broadly similar, though there were some minor differences with respect to the exact position on the gel and the number of components. The second dimension pattern of *Vicia faba* was dissimilar to that of the other species in the number of subunit pairs, having up to 9, most of the minor species having higher molecular weight than the main legumin subunit pairs (Matta et al., 1981).

The legumins of all the members of the *Vicieae* investigated here shared the same electrophoretic behaviour when subjected to SDS-polyacrylamide gel electrophoresis, indicating that all of them are disulphide-bonded subunits, giving a high molecular weight α subunit and a low molecular weight β subunit when analysed by SDS-polyacrylamide gel electrophoresis under reducing conditions (Fig. 3). For all four species almost all the subunits had molecular weights between 60 kDa and 35 kDa, except the *Vicia faba* subunit pattern, which had a number of subunits with molecular weights of more than 60 kDa. Apparently, the subunit patterns of legumins of the four species had two areas of considerable similarity, the first being the region of subunits which had a molecular weight around 55 kDa and the other with a molecular weight around 37 kDa. Under reducing conditions, the subunit pattern of the legumin of each species separated into high molecular weight subunits (α or acidic subunits) and low molecular weight subunits (β or basic subunits). The acidic subunits of all four species were dominated by a number of strongly stained subunits, having Mr between 43 kDa and 33 kDa. On the other hand, the basic subunits were characterized by polypeptides that had molecular weights between 24.5 kDa and 20 kDa.

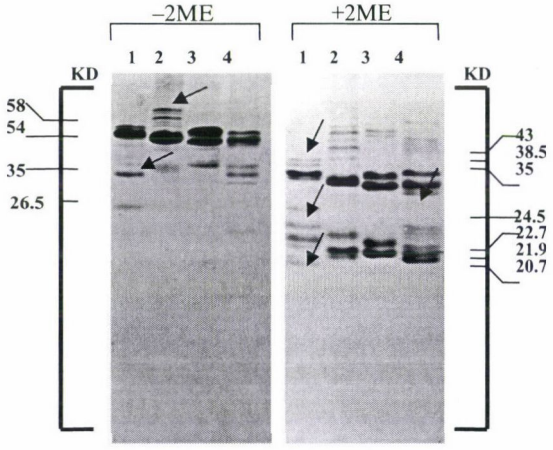



*Fig. 3.* SDS-PAGE of legumins analysed under non-reducing and reducing conditions. 1, *P. sativum*; 2, *V. faba*; 3, *C. arietinum;* 4, *L. esculentum*. The arrows on the left point to bands with Mr between 60 and 35 kDa, on the upper right to bands with Mr between 43–33 kDa, and on the lower right to bands with Mr between 24.5 and 20 kDa

Subunits with Mr 50 kDa, 33 kDa and 14.5 kDa were present in the subunit patterns of the vicilins of all four species (Fig. 4). In the Mr 50 kDa region, the subunit patterns of all four species were similar, except for the vicilin of *Cicer arietinum*, which had one more band with Mr 55 kDa. In the Mr 33 kDa region, the vicilins of *Lens esculentum* and *Cicer arietinum* showed different subunit patterns; the subunit pattern of *Lens esculentum* contained a large number of faint bands, whereas the subunit patterns of vicilin in *Cicer arietinum* had only one strong band that characterized this region. On the other hand, the vicilins of *Vicia faba* and *Pisum sativum* exhibited similar subunit patterns. In the third region, which had molecular weights between 19 kDa and 12 kDa, the subunit patterns showed a clear resemblance for all four species. Generally, it can be concluded that the vicilins of all four species share nearly the same subunit composition, with a few insignificant exceptions.

The electrophoretic patterns of the seed albumins of *Pisum sativum*, *Vicia faba*, *Lens esculentum* and *Cicer arietinum* differed markedly and only one putatively common protein species, corresponding to the major albumin protein of *Pisum*, Mr approximately 25 kDa (Croy et al., 1984), could be identified in the electrophoretic profiles of the different species, with the exception of *Vicia faba* (Fig. 5). However, when the total protein extracts of all four species were examined by double antibody precipitation (Western blotting technique), the Mr 25 kDa band of *Vicia faba* also appeared on the nitrocellulose paper (Fig. 5B), indicating that all four members of the *Vicieae* contain the Mr 25 kDa band. Additionally, all four members of *Vicieae* exhibited electrophoretically and serologically a band that had a molecular weight slightly lower than that of the major albumin of *Pisum sativum*. Quantitatively, this band was present in a very small amount (Fig. 5A).

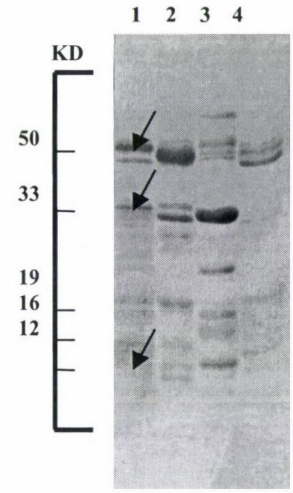



*Fig. 4.* SDS-PAGE of vicilins analysed under non-reducing conditions. l, *P. sativum*; 2, *V. faba*; 3, *C. arietinum*; 4, *L. esculentum*. The arrows point to bands with Mr of 50, 33 and 14.5 kDa

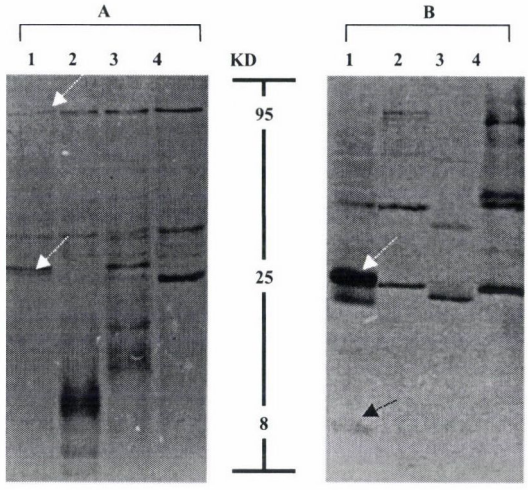



*Fig. 5A.* SDS-PAGE of albumin seed proteins analysed under non-reducing conditions. 1, *P. sativum*; 2, *V. faba*; 3, *C. arietinum*; 4, *L. esculentum.* The arrows point to high molecular weight albumin (110 kDa) and major albumin (25 kDa); *Fig. 5B.* Reaction of albumin seed proteins of *P. sativum, V. faba, L. esculentum* and *C. arietinum* (lanes 1–4) with anti (pea major albumin) IgG after transfer to nitrocellulose and staining with a second antibody (Western blot). The white arrow points to major albumin (25 kDa)

When the total protein extracts, purified legumins and purified vicilins of all four species were allowed to diffuse against *Pisum* anti-legumin antiserum and *Pisum* anti-vicilin antiserum separately in an Ouchterlony double immunodiffusion test, all gave a precipitin arc (data not shown). This indicated that there was a degree of similarity between the total protein extract, purified legumin and purified vicilin of *Pisum sativum* and those of the other members of *Vicieae*. Subsequently, comparative double immunodiffusion was used to study this degree of similarity; the results are shown in Fig. 6, which shows that the legumins of *Vicia faba* and *Lens esculentum* had total identity. In contrast, the legumin of *Cicer arietinum* showed partial identity. Moreover, this work revealed that the vicilins of *Vicia faba, Lens esculentum* and *Cicer arietinum* had partial identity with the vicilin of *Pisum sativum*. However, the degree of this partial identity was less in the case of *Cicer arietinum* (large spur produced) than for the other two species (*Lens esculentum* and *Vicia faba*).

The total protein extracts of *Pisum sativum*, *Vicia faba*, *Lens esculentum* and *Cicer arietinum* were first subjected to electrophoresis in the presence of SDS-PAGE under reducing and non-reducing conditions, then transferred to a nitrocellulose sheet by electroblotting. The immobilized protein on the nitrocellulose sheet was detected with *Pisum* legumin antiserum (Fig. 7) and *Pisum* vicilin anti-serum (Fig. 8). Interestingly, all the members of *Vicieae* which were investigated by the Western blotting technique were found to

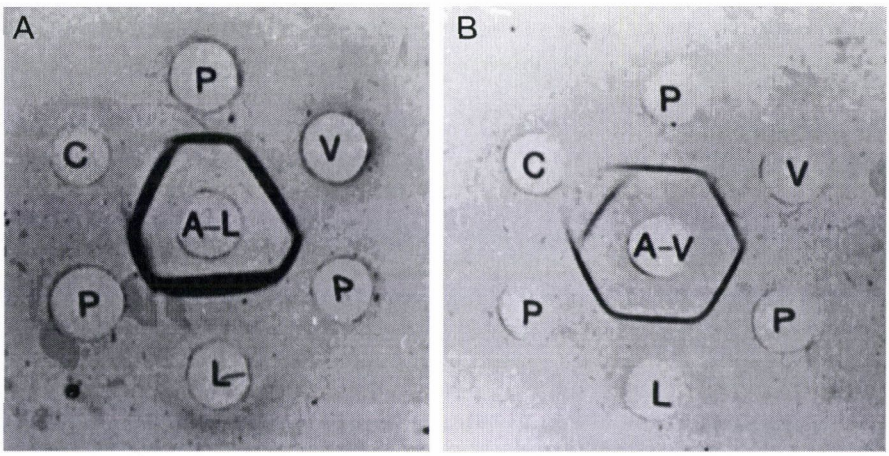



*Fig. 6A.* Immunological reactions of anti (*Pisum* legumin) antiserum (P) against *Pisum* legumin and other proteins; V, *Vicia faba* legumin; L, *Lens esculentum* legumin; C, *Cicer arietinum* legumin; *Fig. 6B.* Immunological reactions of anti (*Pisum* vicilin) antiserum (P) against *Pisum* vicilin and other proteins; V. *Vicia faba* vicilin; L, *Lens esculentum* vicilin; C, *Cicer arietinum* vicilin

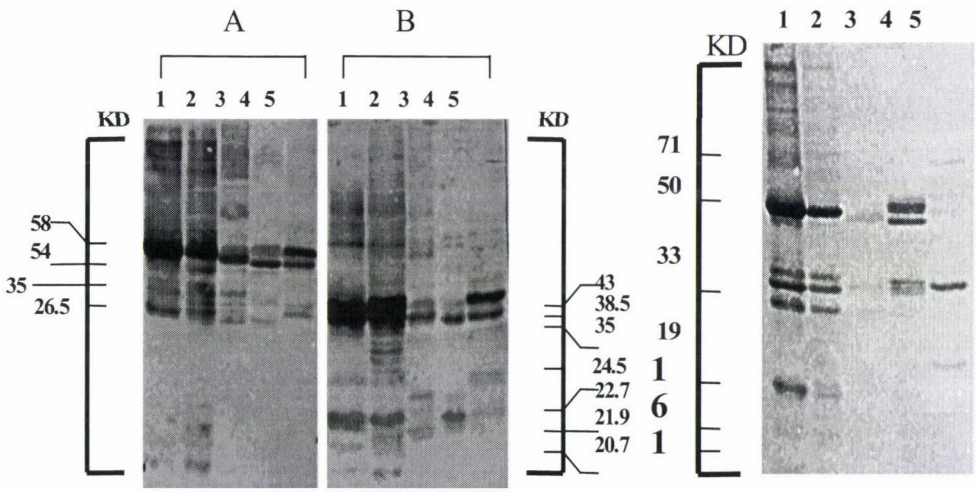



*Fig. 7A.* Reaction of proteins with anti (*Pisum* legumin) IgG after transfer to nitrocellulose and staining with a second antibody (Western blot). 1, *Pisum* total protein extract; 2, *Pisum* legumin; 3, *Vicia* legumin; 4, *Lens* legumin; 5, *Cicer* legumin; *Fig. 7B.* Reaction of proteins with anti (*Pisum* legumin) IgG after transfer to nitrocellulose and staining with a second antibody (Western blot). 1, *Pisum* total protein extract; 2, *Pisum* legumin; 3, *Vicia* legumin; 4, *Lens* legumin; 5, *Cicer* legumin. All tracks were run under reducing conditions

*Fig. 8.* Reaction of proteins with anti (*Pisum* vicilin) IgG after transfer to nitrocellulose and staining with a second antibody (Western blot). 1, *Pisum* total protein extract; 2, *Pisum* legumin; 3, *Vicia* legumin; 4, *Lens* legumin; 5, *Cicer* legumin

contain convicilin-like protein. These convicilins gave a cross reaction with *Pisum* vicilin antiserum on nitrocellulose sheets, having almost the same molecular weight as the convicillin of *Pisum sativum* (Croy et al., 1980). All the subunits of the legumins of *Vicia faba* and *Lens esculentum* gave a very strong reaction under non-reducing conditions with *Pisum* legumin antiserum. However, the subunits of the legumin of *Cicer arietinum* showed a slight difference, in that the lowest molecular weight subunit pair (Mr 33 kDa) failed to react with *Pisum* legumin antiserum under non-reducing conditions. Furthermore, under reducing conditions, three low molecular weight subunits did not react: Mr 22.5 kDa, 21.9 kDa, 21 kDa. When *Pisum* vicilin antiserum was allowed to react with the immobilized total protein of *Pisum sativum*, *Vicia faba*, *Lens esculentum* and *Cicer arietinum* on a nitrocellulose sheet, some subunits of vicilins of *Vicia faba*, *Lens esculentum* and *Cicer arietinum* showed no homology with the vicilin of *Pisum sativum*. In addition, none of the low molecular weight subunits of the vicilin of *Cicer arietinum,* which had molecular weights of 24.5 kDa, 23 kDa, 22.5 kDa, 21.5 kDa and 21 kDa, reacted with *Pisum* vicilin antiserum, whereas some subunits of the vicilins of *Vicia faba* and *Lens esculentum* in the same range of molecular weight reacted. These data explain why the vicilins of *Vicia faba*, *Lens esculentum* and *Cicer arietinum* gave different reactions of identity when investigated with the Ouchterlony double immunodiffusion technique.

The total protein extracts of *Phaseolus coccineus, Phaseolus vulgaris*, *Vigna unguiculata, Dolichos lablab*, *Glycine max*, *Cajanus cajan* and *Canavalia ensiformis* were screened immunologically using the Western blotting technique. Some species gave positive reactions with both *Pisum* vicilin and legumin antisera, e.g. *Canavalia ensiformis;* others gave positive reactions with *Pisum* vicilin antiserum, e.g. *Vigna unguiculata, Dolichos lablab*, *Cajanus cajan;* while some gave positive reactions with *Pisum* legumin antiserum, e.g. *Glycine max* (Table 1).

*Table 1*

Distribution of proteins immunochemically related to either the legumin or vicilin of *Pisum sativum* in seeds of the *Leguminosae* subfamily *Papilionoideae*

| Tribe | Species | Protein detected by anti-*P. sativum* antiserum with the Western blotting technique | |
|---|---|---|---|
| | | V (vicilin) | L (legumin) |
| *Vicieae* | *Pisum sativum* | + | + |
| | *Vicia faba* | + | + |
| | *Lens esculentum* | + | + |
| | *Cicer arietinum* | + | + |
| | *Lathyrus odoratus* | + | + |
| *Phaseoleae* | *Phaseolus coccineus* | – | – |
| | *Phaseolus vulgaris* | – | – |
| | *Vignia unguiculata* | ± | – |
| | *Dolichos lablab* | ± | – |
| *Glycineae* | *Glycine max* | – | ± |
| *Cajaneae* | *Cajanus cajan* | ± | – |
| *Diocleae* | *Canavalia ensiformis* | ± | ± |

+ = strong reaction; ± = weak reaction; – = no reaction

## Discussion

The position of *Cicer arietinum* is still a matter of debate. Some workers support the opinion that *Cicer arietinum* is a member of the tribe *Vicieae*, while others have shown that *Cicer arietinum* should be excluded from *Vicieae* and have its own tribe, the *Ciceridеae*. The tribe *Vicieae* is a member of the subfamily *Papilionoideae* which in its turn is one of four subfamilies of *Leguminosae* (De Candolle, 1825).

Many workers made comparative studies on the members of the tribe *Vicieae*, on the basis of the electrophoreteic patterns of their storage proteins. In these studies, proteins were analysed by non-dissociating gel electrophoresis, in which the position on the gel to which a protein moves is dependent partly on its size and molecular weight and partly on its charge (Boulter et al., 1967). In the light of this fact, taxonomical comparisons based on non-dissociating gel electrophoresis may give misleading conclusions, because it is highly probable that proteins with different molecular weights may have the same electrophoretic mobilities on the gel (Derbyshire et al., 1976). In the present work dissociating gel electrophoresis was used under non-reducing and reducing conditions to compare the storage proteins of members of the tribe *Vicieae*. Reynolds and Tanford (1970) demonstrated that under dissociating conditions all protein specificity was lost and mobility in the gel was a measure of molecular size alone. Therefore, the comparison of seed proteins using dissociating gel electrophoresis is considerably more convincing than the results of non-dissociating gel electrophoresis. However, it is impossible to rely on only one technique to judge whether the taxa under study are homogeneous or not. Using dissociating gel electrophoresis, it was found that the band patterns of the globulin fractions of the total protein extracts of seed meal from *Pisum sativum*, *Vicia faba*, *Lens esculentum* and *Cicer arietinum* were broadly similar. This result conforms with those of Boulter et al. (1967), Jackson et al. (1969) and Hennig and Schlesier (1994), who indicated that the electrophoretic patterns of the globulin major storage proteins of all members of the tribe *Vicieae,* including *Cicer arietinum,* were similar.

The data showed that the electrophoretic patterns of the albumin protein fractions of all four species differed markedly and only one possible common protein species, corresponding to the major albumin protein of *Pisum* (Mr approximately 25 kDa), could be identified (Croy et al., 1984; Gatehouse et al., 1985; Rosa et al., 2000). Though this band was not apparently present in the electrophoretic pattern of *Vicia faba*, it gave a faint reaction with anti-*Pisum* anti-major albumin when investigated using the Western blotting technique. Electrophoretically and immunochemically, all four members of *Vicieae* showed a band with a molecular weight higher than that of *Pisum* major albumin. This band precipitated during *in vitro* synthesis with antibodies raised against the major albumin of *Pisum* (Gatehouse et al., 1985). These data, in conjunction with the findings of Croy et al. (1984), showed that all members of the tribe

*Vicieae* contained major, high molecular weight albumin-like proteins. It also showed that *Lens esculentum* was the most closely related species to *Pisum sativum,* while *Cicer arietinun* and *Vicia faba* had a more distant relationship.

As mentioned in the Results section, the second dimension patterns of *Pisum, Lens* and *Cicer* were broadly similar in terms of the number of subunit pairs, whereas the second dimension pattern of *Vicia faba* was dissimilar to the other species (Matta et al., 1981). On the other hand, the second dimension patterns of all four species exhibited considerable differences on the basis of the number of subunits and their position on the gel. However, the second dimension patterns of the conventional legumin subunits of all four species can be considered similar in the light of the following facts: 1) legumin subunit pairs are divided into three types: the main type (Mr 53–54 kDa) corresponds to the conventional legumin subunit pairs of the Wright and Boulter (1972) model (the major legumin subunits of Casey, 1979), while the other two types (present in lesser amounts) have been designated as "big" (Mr 55–58 kDa) and "small" (Mr 35 kDa) legumin subunit pairs; 2) conventional legumin subunits are synthesized as precursor molecules with a molecular weight of 60 kDa (Croy et al., 1980; Casey et al., 1984; Higgins, 1984; Hager et al., 1995; Jung et al., 1998); and 3) it is possible that the other two types are synthesized as precursor molecules with Mr 80 kDa (Casey et al., 1984; Jung et al., 1998). Though the second dimension gel electrophoresis technique makes it possible to analyse the legumin fraction in total protein extracts without the need for purification, thus saving a great deal of time and relatively high capital outlay, it has two disadvantages which, from our point of view, will limit its use on a large scale in molecular taxonomy. The first is that its effectiveness is associated with the range of distribution of subunit pairs. The second disadvantage is that it is not feasible to analyse multiple samples by this technique on the same gel.

As already stated, legumin was defined as a disulphide-bonded subunit pair and seed storage protein of the globulin type, i.e. salt-soluble but insoluble in water, Mr 300–400 kDa (Wright and Boulter, 1972). This operational definition of legumin was adopted by Matta et al. (1981) and generalized to include all legumin-like proteins by Pernollet and Mosse (1983) and Freitas et al. (2000). On these grounds, the operational definition of legumin is stretched to include the legumins of *Cicer* and *Lens*, since they are seed storage proteins of the globulin type, with a molecular weight of 300–400 kDa; i.e. disulphide-bonded subunit pairs, separated into high molecular weight (α for acidic) subunits and low molecular weight (β for basic) subunits, under reducing conditions. The broad similarity between the legumin composition patterns of all four species supported the opinion that *Cicer* is a member of *Vicieae.*

The electrophoretic patterns of the vicilins of *Vicia faba*, *Lens esculentum* and *Cicer arietinum* were found to consist of two groups of subunits, one with a molecular weight of 50 kDa and the other with a molecular weight of less than 50 kDa, like the vicilin subunit composition of *Pisum.* These different subunits are coded by different gene families and are often subjected to post-translational

modifications (Gatehouse et al., 1983; Slightom et al., 1983). By analogy, it was expected that the vicilins of *Vicia faba, Lens esculentum* and *Cicer arietinum* would have a quaternary structure similar to that of the 7S family. This speculation was supported by the fact that the molecular weights of the vicilins of all four species were almost the same. Though the lowest molecular weight subunits of all four species were electrophoretically similar, immunochemically they did not exhibit any great homology with *Pisum* vicilin anti-serum when tested with the Western blotting technique. However, this did not undermine the suggestion that the vicilins of *Pisum, Vicia, Lens* and *Cicer* are members of the 7S family and are broadly similar. This suggestion was supported by a comparison of the nucleotide sequences, electrophoretic patterns and structural similarities of the vicillins of legume species, in particular to those of the tribe *Vicieae* (Sáenz de Miera and Pérez de la Vega, 1998; Rosa et al., 2000; Tiedemann et al., 2000).

Immunologically, the total protein extracts of all members of the *Vicieae* including *Cicer arietinum,* gave a precipitin arc when they were allowed to diffuse against both *Pisum* legumin and vicilin antisera in an Ouchterlony double immunodiffusion test. Furthermore, both purified legumins and vicilins of all four species showed the same results as well. These data were similar to the data obtained by earlier workers (Kloz and Turkova, 1963; Simola, 1969; Dudman and Millerd, 1975). Though the antibodies used in each work were raised against antigens (legumin and vicilin) of different species (*Pisum sativum, Lathyrus* and *Vicia,* respectively), all these authors demonstrated that all members of the *Vicieae*, including *Cicer*, contained legumin- and vicilin-like proteins. However, there was a discrepancy in the degree of relatedness amongst these proteins. Whereas Kloz and Turkova (1963) and Simola (1969) indicated that all members of the *Vicieae* contained similar legumin and vicilin, Dudman and Millerd (1975) demonstrated that the vicilin-like protein of *Cicer* was quite dissimilar to the vicilin-like proteins of other members of the *Vicieae*, concluding that *Cicer* did not contain vicilin-like protein. It must be borne in mind that this conclusion was reached using the Osserman technique, described as a low sensitivity technique because it was not sensitive enough to detect the third storage protein, later designated as convicilin (Croy et al., 1980). In the present study, the comparative double diffusion technique was used to clarify this point. The resulting data showed that all members of the *Vicieae* had similar or identical legumins, except for *Cicer* legumin, which showed partial identity (small spur produced). On the other hand, the vicilins of *Vicia, Lens* and *Cicer* showed partial identity with that of *Pisum,* but the degree of this partial identity was less than in the case of *Cicer* (large spur produced). It was clear that all members of the *Vicieae* contained legumin and vicilin-like proteins, but neither the legumin nor the vicilin of *Cicer* were in the same order of homology with *Pisum* legumin and vicilin as the other members. These data also indicated that the claim of Dudman and Millerd (1975) that *Cicer arietinum* had no vicilin-like protein was not valid.

As far as it is known, all previous immunochemical studies investigated legumins and vicilins in their native form, not on the basis of their subunit structure. The advent of the Western blotting technique allows such studies to be conducted on the basis of the subunit composition of a specific protein. This technique, therefore, was used in the present work to study the degree of relatedness between different members of the *Vicieae* in terms of the legumins and vicilins of the globulin seed proteins. The data showed that all the legumin subunits of *Vicia faba* and *Lens esculentum* gave a very strong reaction, under both non-reducing and reducing conditions, with *Pisum* legumin antiserum. However, the legumin of *Cicer* exhibited a slight difference, in that the low molecular weight (33 kDa) subunit pairs failed to react with *Pisum* legumin antiserum under non-reducing conditions, and some bands of the basic subunits did not react under reducing conditions. These data confirmed the results obtained using the comparative double diffusion technique, giving a conceivable explanation for the reaction of partial identity with *Cicer* and of identity with the rest of the members of the tribe *Vicieae*. On the other hand, some vicilin subunits of *Vicia*, *Lens* and *Cicer* did not react with *Pisum* vicilin antiserum when the total proteins of the members of the tribe *Vicieae* were immobilized on a nitrocellulose sheet; for instance, all the vicilin subunits of *Cicer* with a molecular weight of less than 25 kDa. These data clarified the reason why *Cicer* exhibited low partial identity (large spur produced), while both *Vicia* and *Cicer* exhibited high partial identity (small spur produced). However, this level of dissimilarity amongst both the 7S family and the 11S globulins of the members of the tribe *Vicieae* did not mean that the structural similarity within each group was at a low level, as reported by Freitas et al. (2000), since the low molecular weight subunits represent a low percentage of each protein species. Interestingly, all the members of the *Vicieae* investigated with the Western blotting technique showed only one band, having nearly the same molecular weight as *Pisum* convicilin, 71 kDa (Croy et al., 1980).

The Western blotting technique was also used to screen representatives of the tribes *Phaseoleae*, *Glycineae* and *Cajaneae* for vicilin- and legumin-like protein. The data revealed, for the first time, that there is sequence homology between *Pisum* globulin and that of some members of the *Phaseoleae* and representatives of both *Glycineae* and *Cajaneae*, in contrast to the results of all previous serological work (Kloz and Turkova, 1963; Dudman and Millerd, 1975). However, these weak homology reactions could be predicted on the basis of known sequence homologies in the proteins (Ko et al., 1993; Garcia et al., 1997; Freitas et al., 2000; Mendoza et al., 2001).

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# INCORPORATION OF LEAF RUST RESISTANCE GENES INTO WINTER WHEAT GENOTYPES USING MARKER-ASSISTED SELECTION

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The breeding and cultivation of resistant wheat varieties is an effective way of controlling leaf rust (*Puccinia triticina* Eriks.). The use of molecular markers facilitates the incorporation of the major leaf rust resistance genes (*Lr* genes) responsible for resistance into new varieties and the pyramiding of these genes. Marker-assisted selection was used to incorporate the *Lr* genes currently effective in Hungary (*Lr9*, *Lr24*, *Lr25*, *Lr29*) into winter wheat varieties. The *Lr* genes were identified using STS, SCAR and RAPD markers closely linked to them. Investigations were made on how these markers could be utilised in plant breeding, and near-isogenic lines resembling the recurrent variety but each containing a different *Lr* gene were developed to form the initial stock for the pyramiding of resistance genes. The results indicate that the marker-assisted selection technique elaborated for resistance genes *Lr24*, *Lr25* and *Lr29* can be applied simply and effectively in wheat breeding, while the detection of the *Lr9* marker is uncertain.

**Key words:** winter wheat, leaf rust, resistance, *Lr* genes, marker-assisted selection

## Introduction

Leaf rust (*Puccinia triticina* Eriks.) is one of the most important leaf diseases of wheat, not only in Hungary, but also worldwide. Pathogen attack leads to both yield losses and a deterioration in technological quality (Barabás and Matuz, 1983). One environmentally sound, low-cost method of control is the breeding and cultivation of resistant wheat varieties. To date, over fifty resistance genes influencing the resistance of wheat varieties to leaf rust (*Lr* genes) have been identified and localised on the wheat chromosomes. The majority of these (29) originate from wild species related to wheat, and some of them are still capable of providing effective protection against pathogen attack in Hungary (e.g. *Lr9, Lr19, Lr24, Lr25, Lr29, Lr35, Lr37*).

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The efficiency of leaf rust resistance genes depends on the composition of the pathogen population. As this changes dynamically over time, new pathotypes virulent to a given *Lr* gene may appear, which means that resistance is not a constant trait. A variety carrying a single resistance gene may become susceptible within a short space of time (Szunics and Szunics, 1995). More durable resistance can be achieved by incorporating a number of *Lr* genes into a single genotype, a process known as gene pyramiding (Nelson, 1978). Traditionally, resistance genes are identified using the relevant rust isolate (Knott, 1989), but this procedure is extremely time, space and labour intensive, and cannot be carried out if no fungus isolate is available or if inoculation is unsuccessful. In many cases the only feasible method for identifying resistance genes is the use of molecular markers (Melchinger, 1990).

Among the molecular markers suitable for the detection of *Lr* genes, reliable, relatively fast and simple analysis can be carried out using those based on the polymerase chain reaction (PCR), such as RAPD, SCAR, STS, CAPS and SSR markers. STS markers closely linked to *Lr9* and *Lr24* were the first to be identified (Schachermayr et al., 1994; 1995). PCR markers are now available for many *Lr* genes (*Lr1, Lr10, Lr13, Lr19, Lr25, Lr28, Lr29, Lr34, Lr35, Lr37, Lr39, Lr41, Lr47, Lr51*; Chełkowski and Stępień, 2001; WheatCAP, 2006). Using reliable DNA markers closely linked to the given *Lr* gene, breeders can select for resistance genes in a segregating population even if no inoculants are available, thus making the selection process simpler and faster. Gene pyramiding, involving the transfer of two or more resistance genes into the same variety, can also be checked using marker-assisted selection (MAS) (Melchinger, 1990; Feuillet and Keller, 1998; Gupta et al., 1999).

MAS can only be used in breeding if there is close linkage between the marker and the resistance gene, leading to a specific PCR reaction clearly demonstrating the presence of the *Lr* gene in materials with diverse genetic backgrounds. Another important criterion is the reproducibility of the reaction. Many authors have reported studies on the reliability of markers linked to various *Lr* genes. The STS markers used to detect *Lr9, Lr19* and *Lr24*, and the SCAR markers linked to *Lr24* and *Lr35* have been shown to give a reliable, simple and relatively rapid indication of the presence of these genes and are thus suitable for marker-assisted selection (Gregáňová et al., 2003; Stępień et al., 2003; Błaszczyk et al., 2004; 2005). This cannot be said of the STS markers for genes *Lr1* and *Lr28*, however, which have not proved sufficiently specific (Błaszczyk et al., 2004; 2005).

Using MAS, the resistance genes *Lr9, Lr24, Lr25* and *Lr29*, which were not previously utilised in Hungary, have been incorporated into Martonvásár varieties with good agronomic and chemical quality traits, well adapted to the climatic and growing conditions of the country. The present experiments were aimed at investigating the suitability of molecular markers for MAS, and at developing lines resembling the recurrent variety, but each carrying a single *Lr* gene for use in the pyramiding of resistance genes for variety development. The results achieved so far are presented in this paper.

## Materials and methods

A backcross programme was begun in the Agricultural Research Institute of the Hungarian Academy of Sciences in 1998, aimed at the transfer of effective *Lr* genes. Martonvásár winter wheat varieties with good agronomic and technological quality parameters, but susceptible or moderately resistant to leaf rust (Mv Emma, Mv Madrigál, Mv Pálma and Mv Magvas) were crossed with near-isogenic lines of Thatcher each carrying a different *Lr* gene (*Lr9*: Transfer/Tc*6; *Lr24*: Tc*6/Agent; *Lr25*: Tc*6/Transec, *Lr29*: Tc*6/Cs7D-Ag#11). The $F_1$ plants were backcrossed to the recurrent parents. Plants carrying markers linked to the *Lr* genes were selected by means of MAS from this first backcross generation ($BC_1$) and from later BC generations, and these were again backcrossed to the recurrent parent. The crosses were carried out in the greenhouse and field.

The choice of *Lr* genes for incorporation was based not only on their effectiveness, but also on whether reliable, closely linked PCR markers were available. These were used for molecular marker-assisted selection in BC generations segregating for the *Lr* genes. The CTAB (cetyl-trimethyl-ammonium bromide) method (Rogers and Bendich, 1985) and the DNeasy® Plant Mini Kit (Qiagen®) were used to isolate DNA. In each combination 3–5 plants were tested in the greenhouse and 10–15 plants in the field. As all four of the tested *Lr* genes are effective in the seedling stage, the leaf rust resistance of the young plants was tested in the greenhouse parallel with the isolation of DNA, in order to monitor the efficiency of marker-assisted selection. The plants were inoculated in the 2-leaf stage with a mixture of leaf rust uredospores collected from varieties with various genetic backgrounds and multiplied in the greenhouse. Six different PCR-based primers were used for the detection of the four *Lr* genes (Table 1).

The PCR reactions were carried out as proposed by the authors cited in Table 1, after which the products were amplified using PTC-100 (MJ Research) and GeneAmp PCR System 9700 (Applied Biosystems) equipment. The reaction products were visible under UV light after electrophoresis on 1.2% agarose gels containing ethidium bromide.

The field leaf rust resistance of the plants (4 donor parents, 4 recurrent parents, BC plants, control: Thatcher) was evaluated in an artificially inoculated nursery. Border rows of a susceptible variety, planted around the tested genotypes, were inoculated in development stage 33–35 on the Zadoks scale (Zadoks et al., 1974) using the uredospore mixture also used in the greenhouse experiments. The spore suspension was injected into the spreader plants using a hyperdermic syringe. The pathogen then spread naturally from these sources of infection. The extent of infection was evaluated in terms of severity (% cover) and reaction type (resistant–susceptible) (Stubbs et al., 1986).

## Results and discussion

The four selected *Lr* genes currently provide complete protection against the leaf rust population in Hungary (Table 2). Data from the literature indicate that these resistance genes also continue to be effective in the rest of Europe (McIntosh et al., 1995; Park and Felsenstein, 1998; Mesterházy et al., 2000). However, their efficiency has not been exploited either in Hungary or in other European countries. One reason for this could be that these genes have been translocated into the common wheat genome from alien species (*Lr9* from *Aegilops umbellata*, *Lr24* and *Lr29* from *Thinopyrum ponticum* and *Lr25* from *Secale cereale*), raising the possibility that chromosome segments having an undesirable effect on yield potential or quality may be incorporated together with the resistance genes. Data from the literature suggest that *Lr9* and *Lr25*

*Table 1*
DNA markers used for marker-assisted selection

| *Lr* gene | Marker | Marker type | Size of amplified product (bp) | References |
|---|---|---|---|---|
| *Lr9* | J13/1, J13/2 | STS | 1100 | Schachermayr et al., 1994 |
| *Lr24* | J09/1, J09/2 | STS | 350 | Schachermayr et al., 1995 |
| *Lr24* | SC-H5/1, SC-H5/2 | SCAR | 700 | Dedryver et al., 1996 |
| *Lr25* | LR25F20, Lr25R19 | SCAR | 1800 | Procunier, 2004 |
| *Lr29* | UBC219 | RAPD | 1000 | Procunier, 1995 |
| *Lr29* | LR29F24, LR29FR24 | SCAR | 900 | Procunier, 2004 |

*Table 2*
Leaf rust resistance of parental varieties included in the crossing programme Martonvásár, 2001–2006

| Genotype | Severity (% cover) and reaction type (R–S) | | | | |
|---|---|---|---|---|---|
| | 2001 | 2002 | 2004 | 2005 | 2006 |
| Mv Emma | 100S | 80S | 100S | 100S | 80S |
| Mv Madrigál | 80S | 60S | 100S | 100S | 60S |
| Mv Magvas | 20MR | 20MR | 30MR | 20MR | 10MR |
| Mv Pálma | 50S | 60S | 60S | 80S | 40MS |
| Transfer/Tc*6 (*Lr9*) | 0 | 0 | 0 | 0 | 0 |
| Tc*6/Agent (*Lr24*) | 0 | 0 | 0 | 0 | 0 |
| Tc*6/Transec (*Lr25*) | 0 | 0 | 0 | 0 | 0 |
| Tc*6/Cs7D-Ag#11 (*Lr29*) | 0 | 0 | 0 | 0 | 0 |

Note: MR: moderately resistant, MS: moderately susceptible, S: susceptible

have a negative effect on yield components and yield level (Ortelli et al., 1996; Procunier et al., 1995). The four Martonvásár varieties included in the MAS programme have good agronomic and technological quality traits, but their leaf rust resistance is unsatisfactory. Mv Pálma, Mv Emma and Mv Madrigál are susceptible, while Mv Magvas is moderately resistant to the pathogen (Table 2). By incorporating resistance genes *Lr9, Lr24, Lr25* and *Lr29* into these varieties, breeding lines could be developed combining good agronomic and chemical quality traits with a known background of leaf rust resistance.

After optimising the PCR reaction conditions (component quantities, cycle number, annealing temperature) all six primers produced amplification products characteristic of the given *Lr* gene in the DNA samples of positive control plants, so it was possible to carry out marker-assisted selection in the segregating generations.

The use of primer J13/1, 2, linked to *Lr9*, proved complicated and the reaction conditions had to be changed at several points before satisfactory products could be obtained. Gupta et al. (2005) also noted problems with this marker pair, making it doubtful whether it can be used for MAS.

The two types of primer used to detect the *Lr24* gene (STS, SCAR) gave well-reproducible reactions and mutually compatible results, i.e. amplified products were found in the same plants with both primers. The method

elaborated for the detection of *Lr25*, involving the LR25F20/R19 SCAR marker pair, was also successfully adapted with minor optimisation to give a reliable reaction.

*Lr29* was first detected using the RAPD primer UBC219, but from 2005 onwards the SCAR primer LR29F24/R24, specific for the *Lr29* gene, was used for MAS. The *Lr29* gene could be detected within a short time with both primers, but the SCAR marker gives a single, clearly identifiable product, making it simpler to apply.

In the field experiments the adult leaf rust resistance of the plants was scored in generations segregating for the *Lr* genes, parallel to MAS (Table 3). In some years the results of the resistance test were uncertain for some plants, either because the pathogen did not spread sufficiently from the susceptible border or because the leaves withered too quickly, before the disease was fully developed (Table 3: +/–/? column). For these plants selection could only be carried out using MAS. In the majority of cases, the results obtained in resistance tests and in MAS were in good agreement, i.e. the markers were identified in resistant plants and not in susceptible ones. For all the primers, however, there were some cases where the results were contradictory: the marker linked to the given *Lr* gene could be detected despite the plants being susceptible (Table 3: +/S column), or the marker could not be detected in resistant plants (Table 3: –/R column). These contradictions could be due to experimental errors in the resistance test, or could indicate that the linkage between the markers and the gene is not complete. The latter could only be tested in a larger population. Whatever the case, these results draw attention to the fact that it is advisable to carry out phenotypic tests on occasion, even when MAS is applied, in order to check the expression of the gene coding the given trait, in the present case leaf rust resistance.

As the result of a backcross programme initiated in 1998, combined with MAS, the genes *Lr9, Lr24*, *Lr25* and *Lr29* were transferred into four selected Martonvásár varieties and various BC generations were produced (Table 4). Plants in the sixth backcross ($BC_6$) progeny generation (*Lr9* combinations and the Madrigál*6//Tc*6/Agent *Lr24* combination) had agronomic traits resembling the recurrent parent and a uniform, homogeneous stand. Tests on various agronomic (height, spike type, kernel hardness, thousand-kernel mass) and chemical quality traits are planned in this generation in order to check whether any undesirable genes with a negative effect on quality or agronomic traits have been introduced into the variety together with the *Lr* genes. Backcrossing and MAS will be continued in the $BC_2$ and $BC_3$ generations.

By pyramiding several *Lr* genes in a single variety, the leaf rust resistance of wheat varieties can be made more durable. The presence of these genes can be monitored using the available DNA markers. Work aimed at pyramiding resistance genes is currently underway in Martonvásár, and $F_1$ plants containing the gene combinations *Lr9+Lr24*, *Lr9+Lr25* and *Lr9+Lr29* are now available.

*Table 3*
Comparison of the results of marker-assisted selection and field resistance tests Martonvásár, 2001–2003 and 2006

| *Lr* gene | No. of plants in the response groups | | | | | Total no. of plants tested |
|---|---|---|---|---|---|---|
| | +/R | +/S | –/R | –/S | +/–/? | |
| *Lr9* | 28 | 1 | 0 | 41 | 8 | 78 |
| *Lr 24* | 39 | 3 | 0 | 19 | 14 | 75 |
| *Lr 25* | 12 | 4 | 3 | 11 | 12 | 42 |
| *Lr 29* | 51 | 7 | 3 | 7 | 24 | 92 |

+: plants containing the marker; –: plants not containing the marker; R: resistant; S: susceptible; ?: uncertain resistance data

*Table 4*
BC generations carrying *Lr* genes, developed in the marker-assisted selection programme Martonvásár, 2006

| Recurrent parents | *Lr* gene | | | |
|---|---|---|---|---|
| | *Lr9* | *Lr24* | *Lr25* | *Lr29* |
| Mv Emma | $BC_6$ | $BC_3$ | $BC_3$ | $BC_3$ |
| Mv Madrigál | $BC_6$ | $BC_6$ | $BC_3$ | $BC_3$ |
| Mv Magvas | $BC_6$ | $BC_3$ | $BC_2$ | $BC_3$ |
| Mv Pálma | $BC_6$ | $BC_2$ | $BC_2$ | $BC_3$ |

In summary, it can be said that the presence of the resistance genes *Lr24*, *Lr25* and *Lr29* in the progeny of crosses can be detected with considerable certainty using PCR markers. The poor reproducibility of the reactions for the STS primer J13/1, 2, linked to the *Lr9* gene, retarded selection, so this primer does not allow effective marker-assisted selection. The new marker recently reported by Gupta et al. (2005) will hopefully solve the problem of detecting the *Lr9* gene in the course of breeding.

With the exception of the STS primer *Lr9*-J13/1, 2, all the primers used in the present work could be useful tools enabling plant breeders to transfer and pyramid *Lr* genes in the course of marker-assisted selection. However, due partly to the novelty of the technique and also to differences in the genetic backgrounds of the wheat genotypes used in breeding, it is still advisable to test the expression of the individual *Lr* genes in field leaf rust resistance tests.

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# ISOLATED MICROSPORE CULTURES OF A HUNGARIAN DURUM WHEAT (*Triticum turgidum* L.) CULTIVAR, MARTONDUR 1

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A number of sporophytically induced microspores and embryo-like structures (ELS) were obtained from isolated microspore cultures of durum wheat (*Triticum turgidum* L. cv. Martondur 1). Various pre-treatments were screened, involving spike treatment at 4°C for 2, 7 or 14 days; anther treatment in 0.4 M mannitol containing macroelements at 33°C for 3 days, and various combinations of these. The frequency of embryogenic (star-like) microspores and the number of ELS showed a very high positive correlation in the cultures. Starvation at high temperature was necessary to achieve a reasonable frequency of microspore embryogenesis. The best results were achieved when starvation at high temperature was combined with no or short (2-day) cold treatment (212±77 and 203±34 ELS/100 anthers, respectively). However, the ELS failed to regenerate; only a few of them produced poorly-developed albino shoots. The present work could be a promising starting point for the production of doubled haploid durum wheat plants in Hungary via isolated microspore culture.

**Key words:** doubled haploid, durum wheat (*Triticum turgidum* L.), isolated microspore culture

**Abbreviations:** DH: doubled haploids, ELS: embryo-like structures

## Introduction

Doubled haploid (DH) plants are important breeding tools in classical cereal breeding (Pauk et al., 1999). DH plants can be obtained by various methods via gynogenesis or microspore embryogenesis from a wide range of cereal species, including barley and bread wheat (Touraev et al., 2001). Since many genotypes of these species show a high androgenic response, efficient barley and wheat breeding programmes are already based on the DH technology (Pauk et al., 1999; Manninen, 1997). Durum wheat, however, seems to be a highly recalcitrant species in haploid cultures.

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Haploid or DH durum wheat plants have already been produced in ovule culture (Mdarhri-Alaoui et al., 1998), ovary culture (Mdarhri-Alaoui et al., 1998; Sibi et al., 2001) and via interspecific pollination (durum wheat × maize: O'Donoughue and Bennett, 1994; Inagaki et al., 1998; Saïdi et al., 1998; David et al., 1999; Knox et al., 2000; Dogramaci-Altuntepe and Jauhar, 2001; Ballesteros et al., 2003; García-Llamas et al., 2004a, b; durum wheat × pearl millet: Inagaki and Hash, 1998; García-Llamas et al., 2004b), but only with unsatisfactorily low efficiency. Despite great efforts, the anther culture technique has not led to a break-through in the efficiency of DH production either. The low frequency of microspore induction, the poor regeneration capacity of the microspore-derived ELS and the extremely high rate of albino plants (around 100 %) were the most serious problems in this culture system (Ghaemi et al., 1992; Otani and Shimada, 1995; Sévenier and Coumans, 1996; Saïdi et al., 1997; J'Aiti et al., 1999; Dogramaci-Altuntepe et al., 2001).

For a long time, no success was achieved using isolated microspore cultures either, but very recently it has proved possible to obtain approx. 6 microspore-derived plants/$10^5$ microspores from several genotypes (Labbani et al., 2005; Cistué et al., 2006), which is an outstanding result.

So far, no significant results have been published regarding DH production from Hungarian genotypes. Therefore, attempts were made to produce DH plants from a Hungarian durum wheat cultivar, Martondur 1, via isolated microspore culture. The protocol of isolated microspore culture adapted to bread wheat (Touraev et al., 1996; Ascough et al., 2006) was applied to durum wheat. The present study screened the effect of pre-treatments, involving the combinations of cold, starvation and heat shock on the frequency of microspore induction, ELS formation and regeneration ability. As a control, isolated microspore cultures were also initiated from the commercial Hungarian wheat cultivar, Mv Pálma.

## Materials and methods

The bread wheat (*Triticum aestivum* L., cv. Mv Pálma) and durum wheat (*Triticum turgidum* L., cv. Martondur 1) plants were grown in phytotron chambers as described by Tischner et al. (1997). Spikes containing late uninucleate and premitotic vacuolated microspores were cut off and subjected to various pre-treatments. In each experiment, one group of five bread wheat spikes was taken as the control with eight groups of durum wheat spikes. Each group contained five spikes. The control wheat spikes were subjected to a 3-day starvation pre-treatment in a solution containing 0.4 M mannitol at 33°C as described by Ascough et al. (2006). Of the durum wheat spikes, one group was given no pre-treatment; three other groups were wrapped in alufoil and incubated at 4°C for 2, 7 and 14 days, respectively. The same treatments (0, 2, 7 and 14 days of cold treatment) were also applied to the other half of the spikes, but the anthers of these latter were subsequently subjected to starvation at 33°C for 3 days. Sixty anthers were dissected from the central part of each spike giving 300 anthers per treatment. These 300 anthers were subdivided into three culture wells (Ø = 30 mm) each containing 1.5 ml medium and 15 ovaries (Hu and Kasha, 1997). Microspore isolation and culture were carried out as described by Ascough et al. (2006), but using A2 induction medium (Touraev et al., 1996) and B5 regeneration medium (Gamborg et al., 1968).

The sporophytic development of the microspores in the cultures was monitored using an Olympus inverted light microscope. The appearance of star-like microspores is a clear reflection of embryogenic induction in the cultures (Indrianto et al., 2001). Five hundred microspores were examined in each 2-day-old culture to determine the frequency of star-like microspores. The number of ELS was also counted in each treatment at day 28. The experiment was carried out in three replications.

## Results

The developmental rhythm and pattern of the control Mv Pálma microspores was practically the same as in previously published studies (Touraev et al., 1996; Indrianto et al., 2001; Lantos et al., 2005; Ascough et al., 2006; Shariatpanahi et al., 2006), so no images are presented.

In responding durum wheat cultures, the sporophytically-induced microspores developed star-like morphology by the end of the pre-treatment or at latest within 2 days of culture (Fig. 1A), as observed in bread wheat. On day 4, optically dense multicellular structures were present in cultures previously exhibiting embryogenic, star-like microspores (Fig. 1B). The frequency of the multicellular structures was very similar to that of the previously observed star-like structures on day 2 (Fig. 1A and B). By the third week of culture, ELS had developed from the multicellular structures (Fig. 1C and D), but these varied greatly in size. At day 28, the ELS were transferred to regeneration medium, but only very few of them developed further, enlarging or producing poorly-developed albino plantlets (Fig. 1E and F).

In the control Mv Pálma cultures, 17.2±3.0% of the microspores showed star-like morphology and 214.7±35.3 ELS/100 anthers were counted (Table 1). Subsequently, 12.6±2.0 green plants/100 anthers regenerated, together with a few additional albino plantlets.

The frequency of microspore induction in Martondur 1 was highly dependent on the pre-treatment applied (Table 1). Practically no star-like (i.e. embryogenic) microspores were observed when no pre-treatment was applied. Cold pre-treatment alone resulted in very low percentages of star-like microspores (below 1%). However, high temperature starvation treatment resulted in a high frequency of star-like structures: on average, 15.2±1.9% of the cultured microspores were embryogenic, which is very similar to that of the control. The combination of short (2-day) cold treatment with high temperature starvation resulted in similarly high microspore induction (14.7±3.3%), but as the duration of the cold treatment increased, the frequency of microspore induction dramatically decreased: the combination of high temperature starvation with 14-day cold treatment resulted in very low sporophytic induction (2.0±0.5% star-like microspores).

The number of ELS/culture exhibited a very strong positive correlation with the frequency of star-like microspores ($r=0.96$). Only the high temperature starvation treatment and the combination of short cold treatment with high

temperature starvation resulted in a similarly high number of ELS (approx. 200/culture; Table 2) as in the control bread wheat variety. Unfortunately, the regeneration capacity of the durum wheat ELS was very weak, in contrast to that of the wheat control, and only a few albino plantlets developed from them.

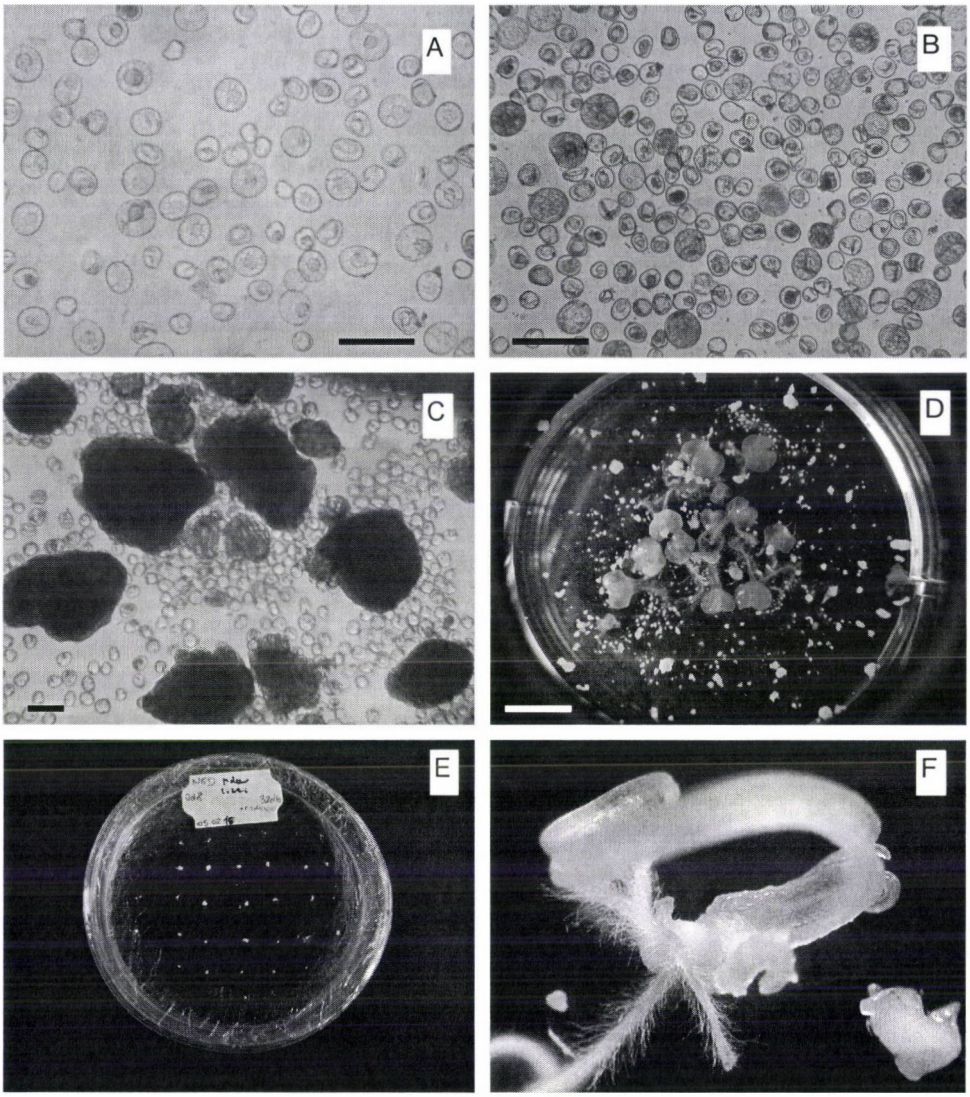



*Fig. 1.* Sporophytic development of durum wheat microspores (cv. Martondur 1) in cultures given starvation pre-treatment at 33°C for 3 days. (A) Microspore population from a 2-day-old culture; note that the enlarged microspores have star-like cytoplasmic morphology. (B) Microspores from a 4-day-old culture; optically dense, multicellular microspores are present at approximately the same frequency as the star-like microspores in the previous stage. (C) Numerous ELS in a 19-day-old culture; note the high diversity in their size. (D) A whole culture of the same age. (E) Plated ELS at day 28. (F) A poorly developed albino plantlet. Bars: 100 μm (A–C), 500 μm (D)

*Table 1*
Frequency of star-like micropores in the 2-day-old cultures

| Pre-treatment | Martondur 1 | | | | | | | | Mv Pálma |
|---|---|---|---|---|---|---|---|---|---|
| Cold (4°C) | None | 2 days | 7 days | 14 days | None | 2 days | 7 days | 14 days | None |
| Starvation (33°C) | None | | | | 3 days | | | | 3 days |
| 1st experiment | 0.0 | 0.0 | 0.0 | 0.8 | 17.4 | 15.1 | 5.6 | 2.4 | 14.4 |
| 2nd experiment | 0.0 | 0.2 | 0.2 | 0.0 | 13.9 | 17.8 | 3.2 | 2.2 | 20.3 |
| 3rd experiment | 0.0 | 0.2 | 0.0 | 0.2 | 14.4 | 11.2 | 6.7 | 1.4 | 16.8 |
| Mean | 0.0[a] | 0.1[a] | 0.1[a] | 0.3[a] | 15.2[c] | 14.7[c] | 5.2[b] | 2.0[a] | 17.2[c] |
| SD | 0.0 | 0.1 | 0.1 | 0.4 | 1.9 | 3.3 | 1.8 | 0.5 | 3.0 |

Mean values followed by the same letter are not significant at p=0.05

*Table 2*
Number of ELS developed from the microspores of 100 anthers in the 4-week-old cultures

| Pre-treatment | Martondur 1 | | | | | | | | Mv Pálma |
|---|---|---|---|---|---|---|---|---|---|
| Cold (4°C) | None | 2 days | 7 days | 14 days | None | 2 days | 7 days | 14 days | None |
| Starvation (33°C) | None | | | | 3 days | | | | 3 days |
| 1st experiment | 0.0 | 0.0 | 0.0 | 0.7 | 278.7 | 187.3 | 17.0 | 8.0 | 177.7 |
| 2nd experiment | 0.0 | 0.3 | 0.0 | 0.0 | 127.3 | 242.0 | 13.7 | 9.3 | 248.0 |
| 3rd experiment | 0.0 | 0.0 | 0.0 | 0.0 | 230.3 | 179.7 | 20.3 | 6.3 | 218.3 |
| Mean | 0.0a | 0.1a | 0.0a | 0.2a | 212.1b | 203.0b | 17.0a | 7.9a | 214.7b |
| SD | 0.0 | 0.2 | 0.0 | 0.4 | 77.3 | 34.0 | 3.3 | 1.5 | 35.3 |

Mean values followed by the same letter are not significant at p=0.05.

## Discussion

The aim of the present study was to induce microspore embryogenesis in a reproducible way from a Hungarian durum wheat cultivar. The sporophytic development of durum wheat microspores was successfully induced, but a reasonable frequency of sporophytic induction was only possible when the microspores in the anthers were subjected to starvation at high temperature. This pre-treatment resulted in a high frequency of star-like microspores, as in the case of hexaploid wheat. The star-like cytoplasmic structure can also be observed in somatic cells at wound sites (Shibaoka, 1994) and in legume roots being prepared for *Rhizobium* infection (Kijne, 1992), which suggest that it may be the consequence of changes in endogenous hormone composition. Hormone levels are key points in androgenesis induction (Gorbunova et al., 2001) and presumably, microspores having the appropriate endogenous hormone levels develop a star-like cytoplasm. Microspores of this type are generally regarded as embryogenic in several species, such as tobacco (Touraev et al., 1997), rape seed (Zaki and Dickinson, 1991) and bread wheat (Touraev et al., 1996; Indrianto et al., 2001; Shariatpanahi et al., 2006). The embryogenic state of these microspores was also confirmed in the present case, as their frequency in isolated microspore culture of durum wheat was similar to that of multicellular

structures and correlated very well with the number of ELS. This high correlation suggests that the pre-treatment affects not only microspore induction but also subsequent embryogenic development.

The simultaneous application of starvation and heat shock pre-treatment proved to be highly efficient for the induction of microspore embryogenesis in bread wheat, but only resulted in a low level of green plant regeneration (Touraev et al., 1996; Table 2). Therefore, cold pre-treatments, which seemed to be successful in the studies of Labbani et al. (2005), were also tested. In the present study, however, as the duration of the cold treatment increased, the number of ELS dramatically declined. When cold pre-treatment was applied alone, the sporophytic induction of microspores was very rare and real embryo-like structures were only obtained at negligible frequencies. These observations are in contradiction to those of Labbani et al. (2005), which showed that five-week cold pre-treatment was the most successful. A direct comparison of these studies is not possible, however, because they were done on different varieties.

The present results, together with other recent findings (Labbani et al., 2005; Cistué et al., 2006), suggest that various combinations of mannitol treatments are the most promising, since this type of starvation usually favoured both microspore embryogenesis and plant regeneration in all the studies made on isolated microspores of durum wheat. Therefore, by studying different combinations of starvation treatments, the genotype dependence of isolated microspore cultures could be overcome. This would result in a widely applicable isolated microspore culture system for durum wheat DH production.

In summary, it can be said that the main bottlenecks in androgenic durum wheat DH production are the failure of microspore induction and the failure of green plant regeneration from ELS. The present work offers a solution to the first of these problems. Together with other recent studies (Labbani et al., 2005; Cistué et al., 2006) the present results show that microspores of durum wheat have very specific requirements for embryogenesis and plant regeneration. Therefore, alternative methods of DH production based on gynogenesis (Mdarhri-Alaoui et al., 1998; Sibi et al., 2001; O'Donoughue and Bennett, 1994; Ballesteros et al., 2003; García-Llamas et al., 2004a, b) are also highly important for this crop.

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# KINETICS OF CADMIUM IN DIFFERENT INDIAN AND HUNGARIAN SOILS: INCUBATION STUDY AT FIELD CAPACITY

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Samples of Hungarian and Indian soils (heavy and light) from different agro-ecological zones were taken in 1998 and 2002, respectively, and the periodic extractability of DTPA-extractable (plant available form) of Cd was determined after incubation at field capacity with different loads of Cd (10, 20, 40 and 80 mg Cd $kg^{-1}$ soil) and correlated with some important soil properties. DTPA-Cd was found to be most highly correlated with cation exchange capacity (CEC), followed by organic matter (O.M.) and pH of the soils. A lower amount of added Cd was recovered in the form of DTPA-Cd with an increase in the time interval.

**Key words**: kinetics, DTPA-Cd, Indian and Hungarian soils, correlation

## Introduction

Soil accumulates toxic heavy metals from a number of diverse sources. These toxic metals reduce microbial activities in the soil, which affects soil fertility at higher concentrations. Metals can directly affect the yields of crops and the quality of food for animals and human beings (Márton, 2006). A knowledge of the dynamics of heavy metals, their transformation into different forms, their retention within an ecological system and their transfer through biological pathways is very important to evaluate the movement of potentially toxic contaminants in the soil. The presence of excess cadmium (Cd) in the soil decreases the yield of crops and also causes nutrient imbalance in the soil. The movement of Cd in the soil is controlled by the status of macronutrients, organic carbon, calcium carbonate, CEC and pH, which play an important role in the formation and precipitation of Cd as less soluble or insoluble phosphates, sulphates, carbonates and other Cd complexes. The plant availability of Cd can be identified by quantifying its release into the soil solution from element

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loadings similar to or exceeding the permissible limit in the wastes applied to agricultural soils and by following its redistribution in the solid and liquid phases under different soil moisture conditions. The availability of Cd to plants may also change with time as it is transformed into various mobile forms. Kádár and Morvai (1998) and Gupta et al. (2000) evaluated the kinetics of Cd in different Hungarian and Indian soils under various agronomic and soil conditions and their interaction with soil properties and the macronutrient status of the soil.

The objective of this study was to evaluate the effectiveness of DTPA extractant to extract Cd (DTPA-Cd; plant available form) at different time intervals from heavy soils [loamy chernozem (H-1), acidic brown forest soil (H-2) and silty clay loam (I-1)] and light soils [calcareous sandy soil (H-3), acidic sandy soil (H-4) and sandy loam (I-2)] from different climatic zones in Hungary and India at field capacity (F.C.).

## Materials and methods

Surface soil samples (0–15 cm) were taken from calcareous clay loam, calcareous sandy soil, acidic brown forest soil and acidic sandy soil in Hungary in 1998 and from sandy loam and loamy sand in India in 2002 and processed. Then all these soils were homogenized with 0.001 M calcium chloride solution and Cd treatments (0, 10, 20, 40 and 80 mg Cd $kg^{-1}$ soil as $CdSO_4$) were applied in triplicate. The F.C. of these soils was maintained by adding distilled water. Five g soil samples were taken from each soil after 1, 3, 7, 14 and 28 days, 50 ml extractant (DTPA-extractant) was added and they were left to soak for half an hour. They were then filtered and analysed for Cd using Atomic Absorption Spectroscopy (Lindsay and Norvell, 1978). All the soils were also analysed for major physico-chemical properties (Table 1).

*Table 1*
Some important physico-chemical properties of the soils (Hungary, 1998; India, 2002)

| Soil code | Soils | pH (1:2 $H_2O$) | < 0.002 mm particle size fraction (%) | O.M. (%) | $CaCO_3$ (%) | CEC [Cmol(p+)/kg] | Cd content (mg $kg^{-1}$) | Origin |
|---|---|---|---|---|---|---|---|---|
| H-1 | Calcareous loamy soil (Chernozem) | 8.1 | 36 | 2.55 | 10 | 20.10 | 0.32 | 1 |
| H-2 | Acidic brown forest soil | 6.8 | 57 | 3.05 | 0.0 | 26.20 | 0.41 | 2 |
| I-1 | Silty clay loam | 8.0 | 32 | 0.55 | 2.50 | 20.20 | 0.10 | 3 |
| H-3 | Calcareous sandy soil | 8.3 | 6 | 0.69 | 15 | 11.35 | 0.22 | 4 |
| H-4 | Acidic sandy soil | 5.4 | 5 | 0.71 | 0.0 | 6.40 | 0.17 | 5 |
| I-2 | Sandy loam | 8.2 | 12 | 0.45 | 1.0 | 12.30 | 0.07 | 6 |

1: Nagyhörcsök Exptl. Station of RISSAC, 1998; 2: Gyöngyös Exptl. Station of Szent István Univ., 1998; 3: Ghaggar belt, Sirsa, 1999; 4: Őrbottyán Exptl. Station of RISSAC, 1998; 5: Nyírlugos Exptl. Station of RISSAC, 1998; 6: RDS Farm, CCS HAU, Hisar, 2002

## Results and discussion

*Heavy soils*

The physico-chemical properties of the experimental soils, which play an important role in the periodic recovery of added Cd, are given in Table 1. The percentage recovery of Cd increased up to 40 mg Cd $kg^{-1}$ in heavy soils (H-1, H-2 and I-1) and thereafter decreased at the 80 mg Cd $kg^{-1}$ level (Table 2).

*Table 2*
Percentage recovery of Cd in Indian and Hungarian soils (Budapest, 2002)

| Soil code | Soils | Cd load (mg $kg^{-1}$) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 10 | 20 | 40 | 80 |
| After a 1-day time interval | | | | | | |
| H-1 | Chernozem | 0.13 | 43 | 61 | 71 | 54 |
| H-2 | Acidic brown forest soil | 0.19 | 38 | 60 | 67 | 42 |
| I-1 | Silty clay loam | 0.05 | 57 | 69 | 83 | 65 |
| H-3 | Calcareous sandy soil | 0.11 | 54 | 78 | 72 | 88 |
| H-4 | Acidic sandy soil | 0.12 | 78 | 79 | 91 | 95 |
| I-2 | Sandy loam | 0.03 | 68 | 89 | 79 | 88 |
| After a 3-day time interval | | | | | | |
| H-1 | Chernozem | 0.13 | 41 | 59 | 69 | 52 |
| H-2 | Acidic brown forest soil | 0.19 | 36 | 69 | 66 | 41 |
| I-1 | Clay loam | 0.05 | 55 | 68 | 81 | 64 |
| H-3 | Calcareous sandy soil | 0.11 | 52 | 77 | 70 | 88 |
| H-4 | Acidic sandy soil | 0.12 | 75 | 78 | 91 | 94 |
| I-2 | Sandy loam | 0.03 | 66 | 87 | 78 | 88 |
| After a 7-day time interval | | | | | | |
| H-1 | Chernozem | 0.13 | 39 | 58 | 65 | 52 |
| H-2 | Acidic brown forest soil | 0.19 | 35 | 60 | 65 | 41 |
| I-1 | Clay loam | 0.05 | 51 | 65 | 80 | 63 |
| H-3 | Calcareous sandy soil | 0.11 | 51 | 73 | 69 | 87 |
| H-4 | Acidic sandy soil | 0.11 | 71 | 77 | 90 | 94 |
| I-2 | Sandy loam | 0.03 | 63 | 85 | 78 | 88 |
| After a 14-day time interval | | | | | | |
| H-1 | Chernozem | 0.12 | 37 | 54 | 64 | 48 |
| H-2 | Acidic brown forest soil | 0.18 | 31 | 68 | 65 | 41 |
| I-1 | Clay loam | 0.04 | 48 | 64 | 79 | 60 |
| H-3 | Calcareous sandy soil | 0.11 | 50 | 73 | 68 | 86 |
| H-4 | Acidic sandy soil | 0.11 | 69 | 76 | 88 | 93 |
| I-2 | Sandy loam | 0.03 | 60 | 82 | 74 | 87 |
| After a 28-day time interval | | | | | | |
| H-1 | Chernozem | 0.11 | 34 | 48 | 62 | 46 |
| H-2 | Acidic brown forest soil | 0.17 | 29 | 68 | 64 | 41 |
| I-1 | Clay loam | 0.04 | 46 | 59 | 75 | 58 |
| H-3 | Calcareous sandy soil | 0.1 | 49 | 70 | 67 | 84 |
| H-4 | Acidic sandy soil | 0.1 | 61 | 74 | 86 | 92 |
| I-2 | Sandy loam | 0.03 | 58 | 79 | 73 | 86 |

Soils with codes H-1, H-2 and I-1 are heavy soils, while H-3, H-4 and I-2 are light soils

By contrast, in light soils (H-3, H-4 and I-2) the Cd level increased up to 80 mg Cd $kg^{-1}$ after a 1-day time interval. The same trend was observed after 3, 7, 14 and 28 days. Due to their high CEC, O.M. and clay content, heavy soils have greater retention capacity at higher loads of Cd as compared to the light soils. The DTPA-extractable Cd decreased at successive time intervals and increased with rising levels of applied Cd. The recovery of Cd from heavy soils ranged from 38–83% of the Cd load on the 1st day and from 29–75% on the 28th day. The corresponding values for light soils were 54–95% and 49–92%. The data further show that the added Cd formed sparingly soluble and insoluble complexes with organic matter, carbonates, sulphates and phosphates (Gupta et al., 2000) to an increasing extent over time.

Figure 1 shows that the amount of recovered Cd decreased over an interval of 28 days in the H-1 soil, ranging from 4.3–3.4, 12.2–9.6, 28.4–24.6 and 43.0–36.5 mg $kg^{-1}$ soil at the 10, 20, 40 and 80 mg Cd $kg^{-1}$ levels, respectively. The corresponding values for the H-2 soil (Fig. 1 B) were 3.8–2.9, 13.9–13.6, 26.8–25.6 and 33.9–32.6 mg $kg^{-1}$ soil, and 5.7–4.6, 13.8–11.7, 33.2–29.9 and 51.7–46.1 mg $kg^{-1}$ soil for heavy Indian soil (I-1, Fig. 1C), which is higher than for the Hungarian soils and may be influenced by the different physico-chemical properties.

*Light soils*

Figure 1 (D and E) shows that in light Hungarian soils the DTPA-extractable Cd ranged from 5.4–4.9, 15.5–13.9, 28.6–26.9 and 70.7–67.1 mg $kg^{-1}$ soil for H-3 and from 7.8–6.1, 15.7–14.8, 36.3–34.5 and 75.9–73.8 mg $kg^{-1}$ soil for H-4 over the 28-day time interval. For the light Indian soil (I-2, Fig. 1F) the values were 6.8–5.8, 17.7–15.8, 31.4–29.0 and 70.7–68.8 mg $kg^{-1}$ soil, which is almost at par with the light Hungarian soils. The same trend was observed in all three light soils from both countries for the 1–28-day time interval. The findings of the present study are in agreement with those of Kádár and Morvai (1998) for Hungarian calcareous soils.

*Correlation of DTPA-Cd with some important soil properties*

The added Cd was found to have a highly significant having fourth order polynomial correlation with CEC ($R^2$= 0.98) and O.M. ($R^2$= 0.98) and a second order polynomial correlation with soil pH ($R^2$= 0.75), as shown in Figures 2, 3 and 4.

## Conclusions

DTPA-Cd was found to be most closely correlated with cation exchange capacity (CEC), followed by the organic matter (O.M.) and pH of the soils. A lower amount of added Cd was recovered in the form of DTPA-Cd with an increase in the time interval. The percentage recovery of Cd was higher in light soils as compared to heavy soils in both countries.

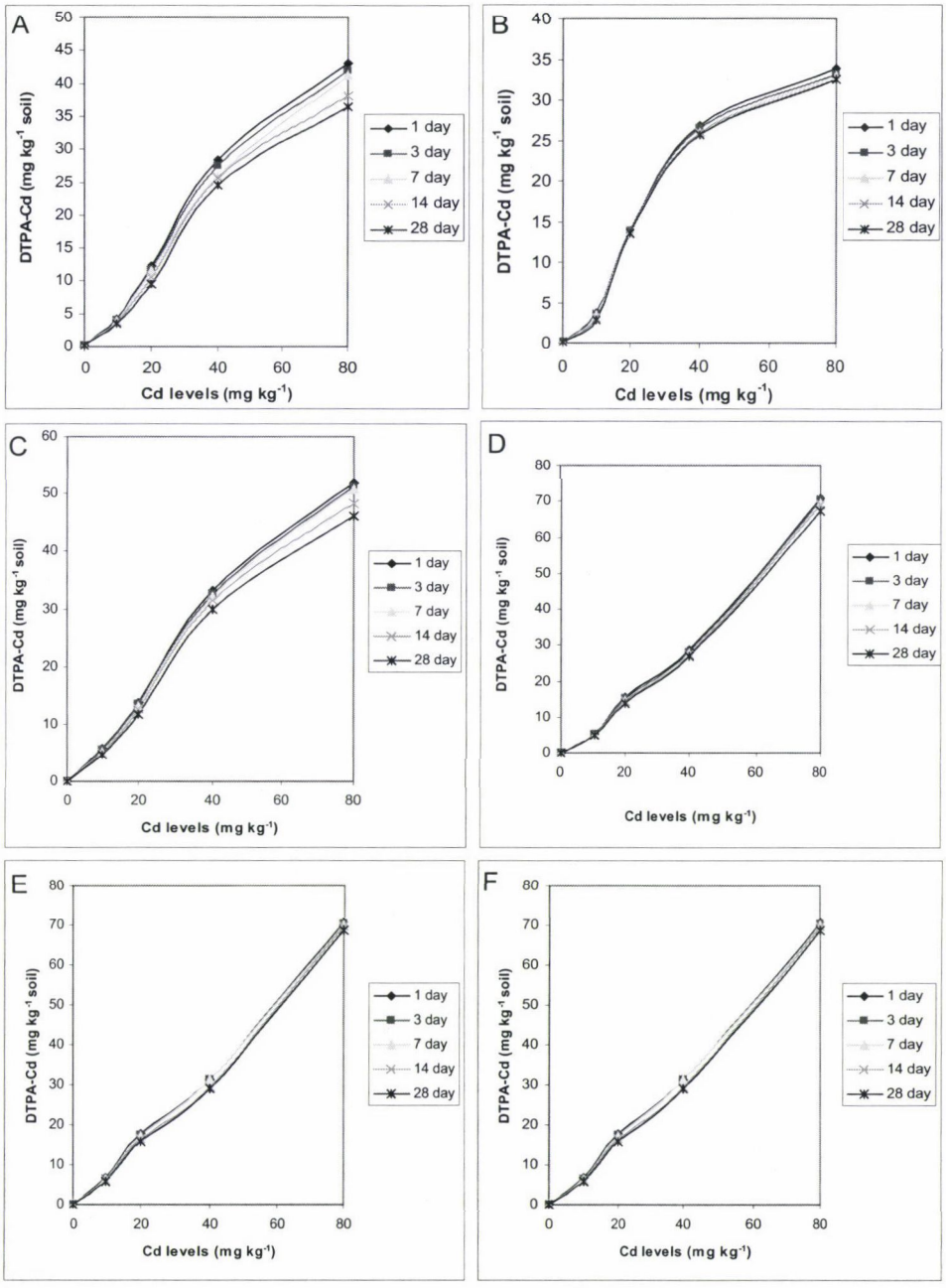



*Fig. 1.* Effect of Cd loadings on DTPA-extractable Cd content in heavy [A (H-1): Calcareous loamy chernozem soil, Nagyhörcsök, Hungary; B (H-2): Acidic brown forest soil, Gyöngyös, Hungary; C (I-1): Silty clay loam soil, Ghaggar belt, Sirsa, India] and light [D (H-3): Calcareous sandy soil, Őrbottyán, Hungary; E (H-4): Acidic sandy soil, Nyírlugos, Hungary; F (I-2): Sandy loam, Hisar, India) soils after 1, 3, 7, 14 and 28 days (Budapest, 2002)

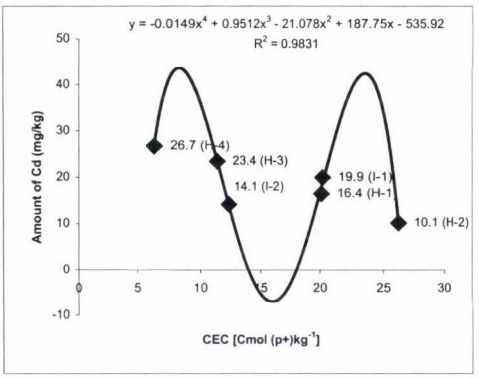



*Fig. 2.* Correlation between soil CEC and DTPA-extractable Cd content after 28 days (Budapest, 2002)

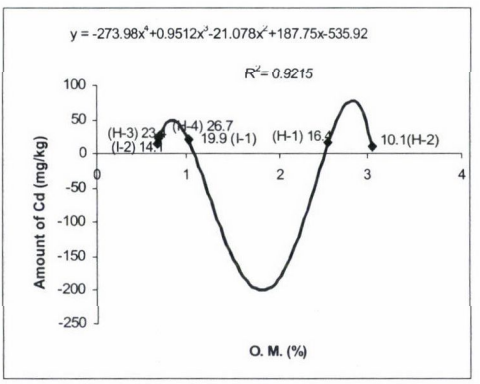



*Fig. 3.* Correlation between soil organic matter and DTPA-extractable Cd content after 28 days (Budapest, 2002)

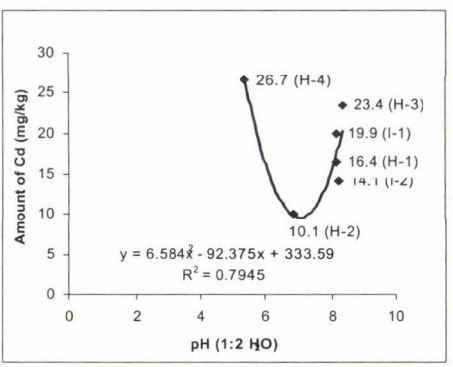



*Fig. 4.* Correlation between soil pH and DTPA-extractable Cd content after 28 days (Budapest, 2002)

## Acknowledgements

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# EFFECTS OF NITROGEN FERTILISATION AND TWO GROWING SITES ON BIOMASS PRODUCTION AND LIGNAN CONTENT OF LINSEED (*Linum usitatissimum* L.): SECOND YEAR

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Mammary and prostatic carcinomas are the most frequently diagnosed in the population of Western Countries. Recent *in vitro* and *in vivo* studies have demonstrated the positive effects of mammalian lignans in retarding the development of these carcinomas. Mammalian lignans, such as enterolactone and enterodiol, originate in the human colon from precursors provided through food consumption. Linseed is currently one of the best sources of secoisolariciresinol (seco) and matairesinol (mata), which have been identified as the major precursors of enterolactone and enterodiol. Even though linseed contains outstanding amounts of these substances, knowledge about the ecophysiology of the active agents is very scarce. Therefore, it seemed necessary to investigate the variability in the formation and accumulation of lignans in linseed in order to provide stable, high-quality plant raw material for the food processing industry. The following paper presents the second growing year of a trial series showing the effects of two locations and enhanced N fertilisation at one site on 12 linseed varieties. Six of the varieties were selected from the previous years and six varieties were included for the first time. It could be shown that the variability of both lignans caused by the factor "cultivar" was much greater than either "location" or "fertiliser intensity". The ranking of the cultivars was very consistent, however, indicating that the genotypic share of the expression was greater than the phenotypic share. The findings confirm the results of the previous year. It was possible to identify superior genetic material based on two growing periods.

**Key words:** secoisolariciresinol, matairesinol, lignan, linseed, nitrogen, *Linum usitatissimum*

## Introduction

Recent studies have shown that mammalian lignans produced in the colon may have anticancer properties (Bingham et al., 1998; Ford, 1999); Adlercreutz, 2002; McCann et al., 2005; Trump, 2005). Mammalian lignans such as enterolactone and enterodiol, which constitute the majority in the human colon, derive from precursors provided through food consumption. There is strong

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evidence that the precursors from plant materials, secoisolariciresinol (seco) and matairesinol (mata), are major sources promoting the development of mammalian lignans (Kulling et al., 1998; Wang et al., 2000). Among the plants screened for seco and mata content so far, linseed seems to contain outstanding contents and is therefore currently by far the best-known dietary source of mammalian lignan precursors (Kurzer et al., 1995; Milder et al., 2005; Niemeyer et al., 2000; Nesbitt et al., 1999; Thompson et al., 1996). However, knowledge about environmental effects on the formation and accumulation of seco and mata in linseed is scarce. Thompson et al. (1996) found significant influences by cultivar and growing conditions, but analysed the products via an *in vitro* fermentation technique rather than their plant precursor contents. Another study found a positive correlation between stress through fungal attack and mata content (Ayres and Loike, 1990). Therefore, it seemed necessary to obtain data on the environmental conditions needed to optimise future linseed cultivation for high lignan yields. Thus, the objective of this study was to verify the findings of the previous trial, indicating that the linseed lignan content was affected more by the cultivar than by the growing conditions. Beyond this, the lignan content stability of the varieties included in the second year of the trial was investigated.

## Materials and methods

*Trial layout*

The experiment was designed either as a 10 × 2 factorial randomised complete block (cultivars and nitrogen fertilisation) in Germany or as a 10 factorial randomised complete block in England. Each treatment was replicated three times. Trial sites were Artern, Germany (51°11' N, 11°17' E) and Roundwood Estates, England (51°21' N, 1°47' W). Ten cultivars were used in the trial, with the cultivars Hella and Oscar sown at only one site each. For a more detailed description of the cultivars used, see Table 1. The levels of the fertilisation factor in Germany were 0 and 60 kg N $ha^{-1}$ (40–60 kg $ha^{-1}$ are recommended for Germany). Plot size was 12.96 $m^2$ (3.6 $m^2$ * 3.6 $m^2$) with 30 rows at both locations.

*Table 1*
Data on the cultivars used in the trial

| Cultivar | Oil content (%) | Seed colour |
|---|---|---|
| No. 1 JT/USA | 38.6 | brown |
| No. 3 Marmalade | 39.1 | yellow |
| No. 5 | 39.9 | yellow |
| No. 6 Scorpion | 39.2 | caramel |
| No. 7 Lirina | 43.3 | brown |
| No. 8 Serenade | 40.6 | brown |
| No. 9 Hella | 37.0 | yellow |
| No. 10 Laser | 39.8 | brown |
| No. 11 Gemini | 38.9 | brown |
| No. 12 Flanders | 36.9 | brown |
| No. 13 Oscar | 37.7 | brown |

The cultivars were selected to obtain material representing a relatively broad genetic range, by picking cultivars from different breeders, or with different properties with regard to seed yield and oil profile. Furthermore, cultivars with different seed colours were selected to meet the different demands of the processing industry for vegetable raw material. From the six varieties tested, the four top-yielding ones in the previous year were selected for the trial, along with six new varieties. So that a broad range of growing conditions could be covered, England was chosen in 2004 rather than the Spanish location which suffered high drought stress in 2003, in order to use a more favourable environment. England is a typically high-yielding area.

Some of the cultivars were obtained from Robin Appel Ltd., Hampshire (GB), while various German breeders provided the rest. At both locations the cultivars were fertilized with ammonium nitrate ($NH_4NO_3$). No target fertilisation was performed, although $N_{min}$ values were determined prior to the trials. In England nitrogen (70 kg N $ha^{-1}$) was applied immediately after sowing on 30 March 2004, and in Germany after 26 d (25 March 2004).

Sowing density was 45 kg $ha^{-1}$ at both sites (approx. 700 seeds $m^{-2}$). Weed control was conducted in Germany by spraying metsulfuron + thifensulfuron twice, and in England by spraying a tank mix of Eagle (amidosulphuron) and Bromolin (bromoxynil). The final harvest took place on 13 Sept. 2004 in Germany and on 17 Sept. 2004 in England after prolonged rainfall. Threshing was performed using a combine, and final cleaning for sample preparation was done by hand.

Throughout the experiment no visible diseases or insect attacks occurred at either location, although Hallmark was applied in England, and Karate in Germany to control cabbage flea beetle. The climatic data of the trial sites is presented in Figure 1.

The English trial was designed as an on-farm experiment, as the plots were put within a linseed field. During the growing period the experimental fields in Germany and England received 315.0 mm and 686.0 mm rainfall, respectively. Daytime temperatures at an elevation of 2 m averaged 13.4°C in Germany and 12.7°C in England over the growing period. Soil properties are shown in Table 2.

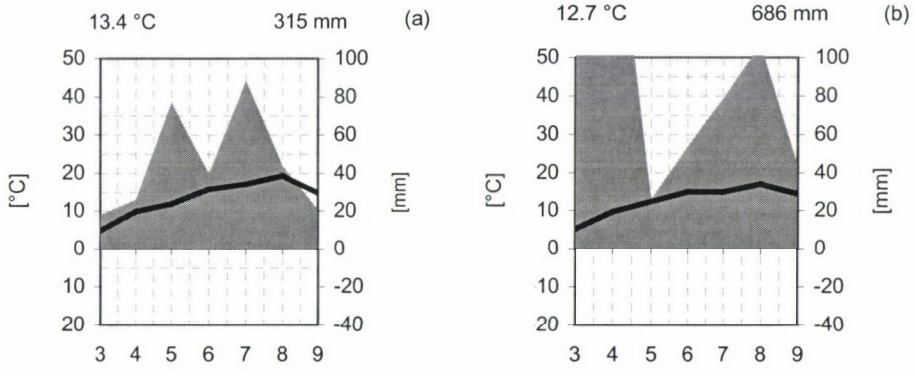



*Fig. 1.* Climatic data of the trial sites in Germany (a) and England (b), (3–9=month; precipitation is shown as a shaded area in mm and average daily temperature as a single dark line in °C)

*Table 2*
Soil parameters of the sites in Germany and England at 0–30 cm depth

| Site | Nutrients | | | pH | Soil texture [%] | | |
|---|---|---|---|---|---|---|---|
| | $N_{min}$ [kg $ha^{-1}$] | P [mg $kg^{-1}$] | K P [mg $kg^{-1}$] | | 2000–63 µm | 63–2 µm | <2 µm |
| Germany | 46 | 250 | 200 | 7.6 | 30.1 | 50 | 19.9 |
| England | 35 | n.d.* | n.d.* | n.d.* | 10 | 60 | 30 |

*not detected

*Determination of lignan content*

*Sample preparation*

2 M hydrochloric acid was added to the milled and defatted linseed samples. The samples were then heated to 100°C and stirred in a Dri-Block® for 2 h. After acid hydrolysis and neutralisation with 8 M sodium hydroxide, the aglycon lignans were extracted with a 1 : 1 mixture of tert-butylmethyl-ether and ethyl acetate (vortex mixer for 1 min). The organic phase was separated by centrifugation. The extraction was repeated twice and the three organic phases combined. After evaporation to 0% moisture, the samples were dissolved in a mixture of pyridine/hexamethyldisilazane/trimethylchlorosilane (9 : 3 : 1) and heated to 60°C for 1 h.

*Gas chromatography*

The lignan standards seco, Anhydr.seco and mata were purchased from Plantech (Reading, UK). For quantification a GC/MS instrument (HP 5890 and quadropole mass spectrometer HP 5972) with a capillary column HP 1 (Dimethyl-Polysiloxan) was used. The temperature of the GC oven was first kept at 100°C for 1 min and then increased by 25°C/min to 300°C. The mass spectrometer was operated in selected ion mode (SIM analysis).

*Statistical analysis*

The data were processed using SPSS (release 12.0, advanced models). A multivariate procedure (GLM) was employed. Yields of seeds and lignans as well as lignan contents were processed for each site separately and over sites. Models of variance for each site and over sites were constructed according to Gomez and Gomez (1984). Correlations were revealed using the Pearson test. For treatment separation of significant factors the Duncan Multiple Range test (varieties) or Tukey test (sites) was applied *a posteriori*. The general level of precision was $\alpha$=5%. Different letters in tables or charts indicate different populations. Error bars in diagrams show the upper confidence limit at 95%.

## Results

### *Seed yield*

Varieties grown at both sites showed 12% higher yields in England than in Germany, although the difference was not significant. The variation in yield was almost half as great in England (0.26 t) compared to Germany (0.49 t). The variety affected only the amount of seeds harvested in Germany (Table 3), whereas no effect was detectable in England. The Serenade variety was the highest yielding in Germany; in England only Oscar (not planted in Germany) performed better than the second-best, Serenade. The stablest variety in terms of seed yield over sites was JT/USA, followed by Laser and Serenade. Only a slight positive effect of the N supply of 190 kg $ha^{-1}$ on the seed yield could be measured over cultivars in Germany ($P< 0.05$). Certain cultivars, such as Flanders > Gemini > Marmalade, performed much better with increasing N supply (on average more than 0.5 t) than e.g. Scorpion, which showed a decrease of 189 kg $ha^{-1}$. The interaction between location and variety was not significant.

*Table 3*
Seed yield [kg ha$^{-1}$] of linseed as affected by growing sites in Germany and England

| Variety | Germany | | England | | | Over sites | |
|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD* | N | Mean | SD |
| No. 13 | – | – | 2250 | 61 | 3 | 2250 | 61a |
| No. 8 | 2086 | 284a | 2180 | 82 | 9 | 2117 | 233ab |
| No. 1 | 1975 | 139a | 2050 | 180 | 9 | 2000 | 147abc |
| No. 5 | 1903 | 308ab | 2077 | 35 | 9 | 1961 | 259abc |
| No. 10 | 1980 | 176a | 1920 | 89 | 9 | 1960 | 149abc |
| No. 7 | 1727 | 208ab | 2153 | 51 | 8 | 1887 | 272bc |
| No. 11 | 1798 | 455ab | 2027 | 72 | 9 | 1874 | 379bc |
| No. 12 | 1790 | 482ab | 2040 | 151 | 9 | 1873 | 408bc |
| No. 6 | 1742 | 341ab | 2127 | 163 | 9 | 1870 | 341bc |
| No. 3 | 1564 | 285b | 2007 | 211 | 9 | 1712 | 333c |
| No. 9 | 1186 | 265c | – | – | 6 | 1186 | 265d |
| Total** | 1843 | 331x | 2064 | 132x | 89 | 1879 | 350 |

*$F$-test not significant; **The site total excludes varieties grown at only one site

*Lignan content*

Both lignan content totals were significantly influenced by the growing site. With the exception of Marmalade and Flanders, all the varieties contained more seco in England than in Germany, resulting in an average of 15% higher content ($P< 0.001$). Correspondingly, for mata only the variety Gemini showed a higher content in Germany. On average the advantage of the English site was 75%, making it much greater for mata than for seco ($P< 0.001$). At both sites the variety Scorpion contained the most seco. In Germany, the difference between Scorpion and all the other varieties tested was significant, whereas in England, Serenade, the second-best, could not be separated statistically from the other varieties. The same was true for the variety Laser, which ranked last with the lowest content, in both England and Germany. It is noticeable that the ranking of the growing sites for each variety with regard to the active agents was more stable than for the seed yield (Fig. 2). This was particularly true for mata, for which the same four varieties (Scorpion and Lirina followed by No. 5 and JT/USA) were the best at both sites, and these four varieties were mainly responsible for the English advantage. Each of the four superior varieties was statistically distinct from the others. Surprisingly, in terms of both seco and mata content, Scorpion, No. 5 and Lirina became the three best varieties over sites. The correlation between seco and mata content showed a medium positive coherence ($r= 0.44$, $P< 0.01$, $n= 80$). Neither active agent was affected by the N supply in Germany.

*Lignan yield*

In contrast to the seed yield, the growing site did affect the lignan yield (Table 4). The total lignan production in England was about 2.5 kg ha$^{-1}$, or 35% higher than in Germany ($P< 0.05$). Of a group of seven statistically equal varieties in Germany, Scorpion and Flanders produced the highest amounts; only

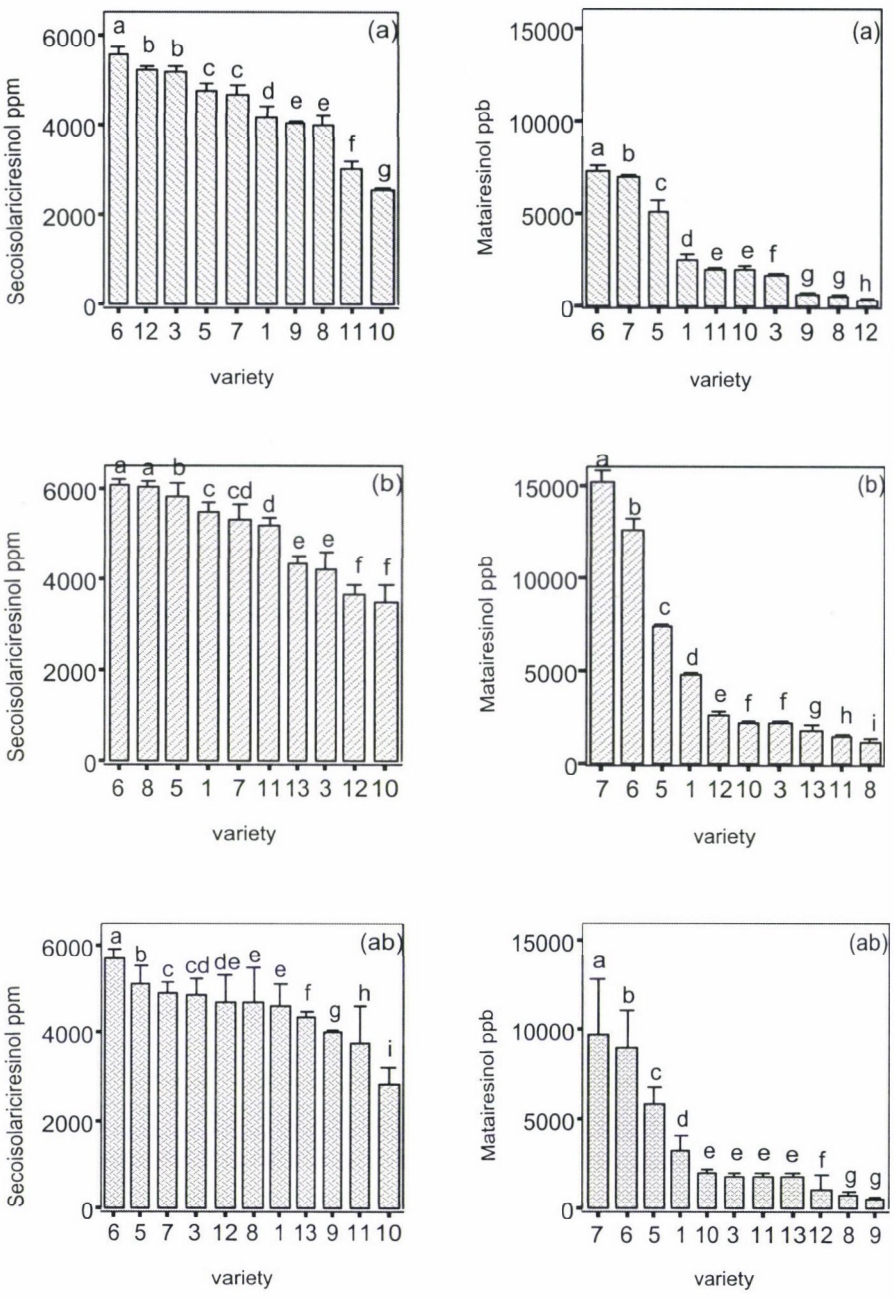



*Fig. 2.* Secoisolariciresinol and matairesinol content as affected by variety and growing site in Germany (a) and England (b) and over sites (ab)

*Table 4*
Lignan yield [kg ha$^{-1}$] of linseed as affected by growing sites in Germany and England

| Variety | Germany | | England | | | Over sites | |
|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | N | Mean | SD |
| No. 13 | 9.08 | 1.82a | 12.00 | 0.98ab | 9 | 10.06 | 2.11a |
| No. 8 | 8.48 | 1.22a | 11.21 | 0.14bc | 9 | 9.39 | 1.67ab |
| No. 1 | 7.87 | 1.22a | 12.25 | 0.47a | 9 | 9.33 | 2.41ab |
| No. 5 | – | – | 9.08 | 0.20e | 3 | 9.08 | 0.20abc |
| No. 10 | 7.53 | 0.74a | 10.64 | 0.15cd | 8 | 8.70 | 1.71abc |
| No. 7 | 7.76 | 0.71a | 10.39 | 0.77cd | 9 | 8.64 | 1.48abc |
| No. 11 | 8.80 | 2.36a | 6.91 | 0.45g | 9 | 8.17 | 2.10bc |
| No. 12 | 7.61 | 1.42a | 7.86 | 1.08f | 9 | 7.69 | 1.25cd |
| No. 6 | 5.17 | 1.45b | 9.80 | 0.45de | 9 | 6.71 | 2.59d |
| No. 3 | 4.72 | 0.41b | 6.21 | 0.54g | 9 | 5.22 | 0.85e |
| No. 9 | 4.48 | 1.00b | – | – | 6 | 4.48 | 1.00e |
| Total* | 7.14 | 2.07x | 9.63 | 2.08y | 89 | 7.98 | 2.38 |

*The site total excludes varieties grown at only one site

Gemini, Laser and Hella were substantially worse. The best varieties in England were Serenade and Scorpion together with No. 5, which ranked third but, like Serenade, could not be separated statistically from Scorpion. Over all sites Scorpion, No. 5 and Serenade were the top three in decreasing order. It can be seen that, due to the homogeneous seed yield results in England, the lignan yield was influenced to a much greater extent by the lignan contents (change in $R^2$: lignan contents 0.94 $\Rightarrow$ seed yield 0.06) than by the seed yields. This effect was much reduced but still extant in Germany (change in $R^2$: lignan contents 0.5 $\Rightarrow$ seed yield 0.48). Two-thirds of the lignan yield is therefore determined by the lignan contents over sites.

## Discussion

At both growing sites the seed yields in 2004 were substandard compared to average yields for the previous years. The yield formation of linseed depends on the number of seeds/m$^2$, which is determined by the number of capsules and the number of seeds per capsule (Pörksen, 1991). Therefore, the linseed yield formation depends principally on the number of branches in the upper part of the plant, as linseed has terminal blossoms, which were very poor this year. It is very likely that this predisposition was suppressed by the climate and therefore the number of blossoms was reduced. Even though more rain fell during the trial period than in the year before, it still remained suboptimal, with roughly 100 mm less than the recommended amount during the vegetation period in Germany, which was reinforced by the slightly worse growing area compared to 2003 with regard to the groundwater level. Furthermore, a long period of cold nights in late spring could have been partly responsible for reduced yields. In England the lower yields could have been due to the exceptionally hot weather during early flowering, which might have led to partial drought stress and reduced seed set

(Cross et al., 2003). However, thousand-seed weight (TSW) was normal to good in Germany (5.9 g). In England the yields consisted of significantly heavier seeds (6.6 g), which was probably due to sufficient rainfall during the later seed-filling phase. In England, seed yield and TSW showed a positive correlation ($r = 0.52$, $n = 30$, $P = 0.03$).

It is known that linseed is capable of mobilising large amounts of mineralised N from the soil without showing great response to N fertilisation. It is very likely that the good $N_{min}$ values in early spring in Germany restricted the advantage of additional nitrogen to only 190 kg seed/ha over all varieties.

No information is available on the ecophysiology of lignan formation except for the results of former trials in the present program (Zimmermann et al., 2006) and the data reported by Westcott et al. (2002). In general the present findings confirm the previous results. As in 2003, no response to increased N supply could be detected, which was probably due to the $N_{min}$ supply at the trial sites being sufficient for linseed. Further investigations are needed for a conclusive clarification. The trial site, again, showed significant effects of the more favourable conditions in terms of higher yields, perhaps indicating an environmental dependency other than the N supply. Matairesinol was, again, more variable in accordance with growing conditions. However, as in 2003, the strongest effects were caused by genetic predisposition. Even though the degree of genetic influence varies among cultivars, it was noteworthy that varieties grown in Germany in both years (Scorpion, No. 5, Marmalade, JT/USA) had almost identical lignan contents in 2003 (seco 4904 ppm; mata 4524 ppb) and 2004 (seco 4914 ppm; mata 4118 ppb). In particular, the cv% of Scorpion for seco content was only 5.7 over three sites and two years, whereas the seed yield varied by 20.7% (Table 5).

*Table 5*
Mean and coefficient of variation of seed yield [kg $ha^{-1}$] and content of secoisolariciresinol [ppm] and matairesinol [ppb] over sites and years

| Variety | N | Year | Site | Seed yield | | Seco | | Mata | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Mean | cv% | Mean | cv% | Mean | cv% |
| JT/USA | 27 | 2 | 4 abcd | 1665 | 40 | 4353 | 16 | 3432 | 22 |
| Marmalade | 27 | 2 | 4 abcd | 1466 | 47 | 3957 | 20 | 2370 | 33 |
| No. 5 | 27 | 2 | 4 abcd | 1706 | 38 | 4913 | 13 | 5319 | 16 |
| Laura | 18 | 1 | 2 ab | 476 | 48 | 2604 | 11 | 2940 | 39 |
| Bilton | 18 | 1 | 2 ab | 1648 | 51 | 3209 | 13 | 3284 | 13 |
| Scorpion | 18 | 2 | 3 bcd | 2083 | 21 | 5566 | 6 | 8237 | 24 |
| Serenade | 9 | 1 | 2 cd | 2117 | 11 | 4688 | 22 | 738 | 41 |
| Laser | 9 | 1 | 2 cd | 1960 | 8 | 2850 | 17 | 2012 | 11 |
| Gemini | 9 | 1 | 2 cd | 1874 | 20 | 3759 | 29 | 1799 | 17 |
| Flanders | 9 | 1 | 2 cd | 1873 | 22 | 4707 | 17 | 1078 | 104 |
| Lirina | 8 | 1 | 2 cd | 1887 | 14 | 4894 | 7 | 9688 | 42 |
| Hella | 6 | 1 | 1 c | 1186 | 22 | 4020 | 1 | 605 | 13 |
| Oscar | 3 | 1 | 1 d | 2250 | 3 | 4343 | 1 | 1793 | 5 |

a Spain 2003: Average 18.7°C, 166 mm; b Germany 2003: Average 14.5°C, 197 mm; c Germany 2004: Average 13.4°C, 315 mm; d England 2004: Average 12.7°C, 686 mm

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# EFFECTS OF *Glomus mosseae* STRAINS OF DIFFERENT ORIGIN ON PLANT MACRO- AND MICRONUTRIENT UPTAKE IN CD-POLLUTED AND UNPOLLUTED SOILS

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In the present study, changes in the infectivity and effectiveness of four *Glomus mosseae* strains of different origin were investigated in calcareous loamy chernozem soils treated with Cd at three levels (0, 50, 100 mg Cd $kg^{-1}$) in a pot experiment. Frequency of infection (F%), arbuscular richness (a%) and shoot dry matter, macro- (N, P, K, S, Ca, Mg) and microelements (Zn, Cu, Ni, Mn, Mo, Co) and the Cd content of the host plants were compared to determine whether there was any variability in infectivity and effectiveness between *G. mosseae* strains of different origin. Functional diversity was found in the infectivity and effectiveness of the studied *G. mosseae* strains. In Cd-treated soil, AMF inoculation was beneficial to the plant growth, P uptake and shoot Cd content of the host. However, the higher uptake of other macro- and microelements was noted for non-mycorrhizal plants compared to mycorrhizal plants. The lower shoot content of some elements did not cause nutrient deficiency in mycorrhizal plants. The present results support the hypothesis that in polluted soils, the development of mycorrhizal symbiosis has the potential for AMF to protect their hosts against Cd toxicity rather than to improve nutrient uptake.

**Key words:** arbuscular mycorrhizal fungi, Cd stress, macro- and micronutrient concentration, functional diversity

## Introduction

Arbuscular mycorrhizal fungi (AMF) are an abundant and functionally important group of soil microorganisms. They can form symbiotic associations with more than 80% of terrestrial plant species. The role of AMF in natural and agricultural ecosystems has been investigated in detail. AMF diversity is found to be in positive correlation with plant diversity and stability (van der Heijden et al., 1998). The importance of AMF colonization in enhancing plant productivity may be greater in disturbed habitats. The ability of AMF to improve the mineral nutrition of the host plant has also been demonstrated (Clark and Zeto, 2000).

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The extensive extraradical hyphal network around the root enhances the absorption surface for nutrients and water. Therefore, AMF may enhance plant growth and the ability of plants to tolerate biotic and abiotic stress conditions (pathogen, nutrient imbalances, drought and heavy metal stress) (Leyval et al., 1997; Takács et al., 2005).

In contaminated soils, the availability and toxicity of metals to plants can be modulated by microbial activity in the rhizosphere including AM fungi (Adriano et al., 2004). A decrease in the heavy metal content of the shoot has been observed due to mycorrhizal colonization (Gildon and Tinker, 1983), while others have found higher metal concentration in mycorrhizal plants (Tonin et al., 2001). Although the results of experiments carried out on sites contaminated with heavy metals are variable and not free from contradictions, AMF appear to be capable of alleviating the phytotoxicity caused by heavy metals. The specific biochemical and molecular backgrounds of AMF adaptation and tolerance to high heavy metal loads are poorly understood. Hutchinson et al. (2004) hypothesized that the lower Cd concentration of AM plants may be caused by fungal immobilization. Others have shown a dilution effect due to the potential of mycorrhizal inoculation to facilitate plant growth under heavy metal stress conditions (Chen et al., 2004). Because of the abilitiy of AMF colonization to alleviate phytotoxicity, the application of AMF inocula may be a promising strategy in phytoremediation (Takács et al., 2005; Biró and Takács, 2005).

Most studies have concentrated only on the uptake of nutrients (N, P, Zn, Cu) and heavy metals as pollutants by mycorrhizal plants. Little is known of changes in the uptake of other important macro- and micronutrients by these plants (Pacovsky, 1986; Raju et al., 1990). Moreover, investigations are generally carried out only with one or a few AMF species and little information is available about intraspecific functional diversity. The objective of this study was to investigate changes in the infectivity and effectiveness of mycorrhizal plants inoculated with different *Glomus mosseae* strains compared to non-mycorrhizal plants in soils treated with Cd at three levels.

## Materials and methods

The soil originating from Nagyhörcsök (Hungary) is classified as a calcareous loamy chernozem with the following characteristics: $pH_{(H2O)}$: 8.17; $pH_{(KCl)}$: 7.32; $CaCO_3$ content: 7.66 %; humus content: 3.48 %. Soil samples were mixed with sand in a 2:1 (w/w) ratio and sterilized (autoclaving twice at 120 kPa for 1–1.5 h). Cadmium sulphate solution (60 ml, 3 $CdSO_4$ . 8 $H_2O$) was added to the soils at three levels (0, 50, 100 mg metal $kg^{-1}$ dry soil).

The S, HS and M strains of *Glomus mosseae* tested (Nicholson and Gerdemann, 1968) originated from various soil types and geographical areas of Hungary. Spores of the *G. mosseae*-M culture were isolated from a calcareous loamy chernozem soil polluted with cadmium (90 mg Cd $kg^{-1}$ soil) in an 8-year long-term field experiment in Nagyhörcsök (Kádár, 1995). This *G. mosseae* culture is probably a Cd-adapted strain. The *G. mosseae*-S inoculum originated from a salt-affected soil (Nyírőlapos) and the *G. mosseae*-HS inoculum from a humus sandy soil (Őrbottyán), and were grown from single spores (Takács and Vörös, 2003a). The *G. mosseae*-BEG12 inoculum

originated from the European Bank for *Glomeromycota* (http://www.kent.ac.uk/bio/beg). The fungal inoculum was a mixture of dried calcareous loamy chernozem soil, spores, mycelium and strongly colonized (F%= 95–100; a%= 65.22–70.85) root fragments of white clover. A quantity of 30 g AMF inoculum (5 w/w%) per pot was used in all the mycorrhizal treatments. Non-inoculated plants were used as control in each treatment. The control plants received a 30 g mixture of non-infected roots of white clover and dried soil. White clover (*Trifolium repens* L.) was grown in pots (0.15 g seed $pot^{-1}$) for two months in a growth chamber under controlled climatic conditions with a temperature of 15–17/23–25°C and a 8/16 h dark/light (25000 lux) period. The white clover seeds were sterilized with ethyl alcohol (70%) for 20 s and washed with distilled water (6 times). The plants were watered every two days. After harvesting, a small part of the root samples was stained with analine blue as described by Phillips and Hayman (1970). Mycorrhizal parameters such as frequency of infection (F%) and arbuscular richness (a%) were estimated using the five-class system according to Trouvelot et al. (1986). The dry weights of shoots and roots were measured after drying at 80°C for 10 h. The element contents of the plant samples were measured after wet digestion with concentrated $HNO_3$ + $H_2O_2$ by inductively coupled plasma emission spectrometry (ICP-AES: JY Ultima2, Jobin Yvon, Villeneuve d'Ascq, France). The cadmium and microelement concentrations (mg $kg^{-1}$) and macroelement contents (w/w%) of the shoots were recorded. The total and available Cd contents in the soil, extractable using the ammonium-acetate + EDTA method, were determined (Lakanen and Erviö, 1971). The soil AL (ammonium-lactate)-soluble $K_2O$ and $P_2O_5$ contents were measured according to Sarkadi et al. (1965) and Egner et al. (1960). The original P supply of the soil was weak, while the K supply was medium. The KCl-exchangeable $NH_4^+$-N and $NO_3^-$-N contents of the soil were determined as described by Bremner and Keeney (1966). All treatments were performed in triplicate (Table 1a, b).

The data are presented as means of replications with least significance differences (LSD) and were statistically evaluated by analysis of variance ($P < 0.05$). F tests were used to test the treatment effects on the measured parameters.

*Table 1a*
Original macronutrient contents of the experimental soil

| Macronutrients | Element content in soil (mg $kg^{-1}$) |
|---|---|
| AL-$K_2O$ | 166 |
| AL-$P_2O_5$ | 84.5 |
| KCl-$NH_4$-N | 2.39 |
| KCl-$NO_3$-N | 4.09 |

*Table 1b*
Content of cadmium (mg $kg^{-1}$; total and available) in soil treated with Cd at three levels

| | 0 mg $kg^{-1}$ Cd | 50 mg $kg^{-1}$ Cd | 100 mg $kg^{-1}$ Cd |
|---|---|---|---|
| Total | 0.126 | 50.50 | 105 |
| Available | 0.081 | 44.54 | 92.24 |

## Results and discussion

The frequency of infection (F% values) of *G. mosseae* strains of different origin were not affected by increasing the Cd content of the soil (Takács and Vörös, 2003b) except for *G. mosseae*-BEG12, where the infection was reduced significantly at the highest Cd rate (Table 2a, b). Significant differences between the various *G. mosseae* strains could be observed in the values of a% at each

level of Cd contamination in the soil. The arbuscular richness of the *G. mosseae*-S and -HS strains isolated from non-polluted soils was enhanced by increasing Cd application, while the a% values of the *G. mosseae*-M strain, isolated from Cd-polluted soil, decreased in Cd-treated soil.

The reduction in shoot dry matter was significant in all mycorrhizal and non-mycorrhizal treatments with increasing Cd application, but the biomass production of the mycorrhizal plants was significantly higher than that of non-mycorrhizal plants at each Cd level, which is in agreement with the results of Chen et al. (2004). Inoculation with various AMF strains resulted in significant changes in shoot biomass at each Cd application rate. A weak correlation was found between the plant growth response and the mycorrhizal parameters (F%, a%) of the *G. mosseae* isolates. The greatest alleviation of the Cd-induced growth reduction was shown in the case of the *G. mosseae*-S and -HS treatments even at the highest Cd rate (100 mg Cd $kg^{-1}$ soil) (Table 2a, b).

The shoot cadmium (Cd) content of mycorrhizal and non-mycorrhizal plants increased with increasing soil Cd content. Significant differences in the shoot Cd concentration of mycorrhizal plants were observed at each level of Cd application. Moreover, significant differences were observed in the effects of the various *G. mosseae* isolates on the shoot Cd content in Cd-loaded soils. The non-Cd-adapted *G. mosseae* strains (*G. mosseae*-S and -HS) were more efficient in reducing the shoot Cd concentration compared to the presumably Cd-adapted *G. mosseae*-M strain. The spores of the *G. mosseae*-M culture were isolated from Cd-loaded soil in a long-term field experiment, so this strain was assumed to be tolerant of Cd after eight years of pollution. The shoot Cd concentration of mycorrhizal plants was only lower than that of non-mycorrhizal plants in the highest soil Cd treatment (100 mg Cd $kg^{-1}$ soil) (Table 2a, b), in agreement with other observations (Gildon and Tinker, 1983; Takács and Vörös, 2003b).

One of the most important impacts of AMF inoculation on the nutrition of the host is the enhancement of P uptake. The shoot phosphorus (P) content (%) was significantly higher in most cases in mycorrhizal plants inoculated with various *G. mosseae* strains compared to that of non-mycorrhizal plants even when Cd was present in the soil (Riverra-Becerril et al., 2002; Parádi et al., 2003) (Table 3a, b). In the present experiment, a significantly higher P content was measured in the shoots of plants colonized with the *G. mosseae*-M culture both at low and high soil Cd levels than in the shoots of plants inoculated with the *G. mosseae*-BEG12, -H and -HS strains. Significant differences were not observed between the shoot P contents of plants colonized with *G. mosseae*-BEG12, -H and -HS in either unpolluted or polluted soils.

*Table 2a*
Infectivity (F%, a%) of the different *Glomus mosseae* in soils treated with Cd at three levels

| AMF treatment | 0 mg Cd $kg^{-1}$ soil | | 50 mg Cd $kg^{-1}$ soil | | 100 mg Cd $kg^{-1}$ soil | |
|---|---|---|---|---|---|---|
| | F% | a% | F% | a% | F% | a% |
| *G. mosseae*-BEG12 | 100 | 12.78 | 98.34 | 13.25 | 11.67 | 0 |
| *G. mosseae*-S | 95.56 | 34.88 | 100 | 40.77 | 97.78 | 54.18 |
| *G. mosseae*-M | 100 | 62.96 | 98.89 | 36.99 | 100 | 40.03 |
| *G. mosseae*-HS | 96.55 | 26.39 | 100 | 37.51 | 100 | 42.88 |
| $LSD_{5\%}$ | 3.63 | 17.17 | | | | |

*Table 2b*
Shoot dry matter (g $pot^{-1}$) and shoot Cd concentration (mg $kg^{-1}$) of mycorrhizal and non-mycorrhizal white clover (*Trifolium repens* L.) plants in soils treated with Cd at three levels

| AMF treatment | 0 mg Cd $kg^{-1}$ soil | | 50 mg Cd $kg^{-1}$ soil | | 100 mg Cd $kg^{-1}$ soil | |
|---|---|---|---|---|---|---|
| | Shoot dry matter | Shoot Cd content | Shoot dry matter | Shoot Cd content | Shoot dry matter | Shoot Cd content |
| Control | 1.88 | 0.15 | 1.00 | 1.89 | 0.78 | 4.34 |
| *G. mosseae*-BEG12 | 3.05 | 0.09 | 1.76 | 3.19 | 1.62 | 3.94 |
| *G. mosseae*-S | 3.74 | 0.62 | 3.48 | 2.04 | 2.69 | 2.59 |
| *G. mosseae*-M | 2.12 | 0.10 | 1.36 | 2.48 | 1.29 | 3.39 |
| *G. mosseae*-HS | 3.62 | 0.13 | 3.29 | 1.32 | 2.70 | 2.44 |
| $LSD_{5\%}$ | 0.66 | 1.17 | | | | |

Data are means of 3 replications ($P<0.05$). *G. mosseae*-BEG12: obtained from the European Bank of *Glomeromycota*; *G. mosseae*-S: spores isolated from salt-affected soil; *G. mosseae*-M: spores isolated from Cd-polluted soil; *G. mosseae*-HS: spores isolated from humous sandy soil

Lower, equal and higher shoot N% values were recorded in AM plants compared to non-AM white clovers grown in Cd-treated soil at the three levels (Table 3a, b). The increased N uptake of AM plants is usually caused by higher N demand due to enhanced P uptake and by the direct effect of AM colonization. Higher uptake of both N forms is also often reported for AM fungi (Clark and Zeto, 2000; Johansen et al., 1994).

The experimental evidence for the uptake of macronutrient cations by AM plants is lacking or inconsistent, especially in polluted soils. The uptake of potassium (K), magnesium (Mg) and calcium (Ca) were lower in AM plants than in non-AM plants grown in neutral or alkaline soils (Raju et al., 1990; Bethlenfalvay and Franson, 1989). The K content was found to be only slightly affected by either AMF treatment or Cd application. The shoot K content was significantly higher in non-AM plants than in AM plants. Large differences were noted for shoot K content between the various *G. mosseae* isolates. Similar results were found for Ca uptake. In contrast to K and Ca uptake, hosts colonized with the infective *G. mosseae* strains (*G. mosseae*-S, -HS, -M) had significantly higher shoot Mg content than non-AM plants at each rate of Cd application. However, the shoot Mg content did not change with increasing soil Cd rate (Table 3a, b).

Little is known of the role of AMF in the uptake of sulphate. Enhanced S uptake was found in both mycorrhizal and non-mycorrhizal plants with increasing soil Cd content. The shoot S content of AM plants was higher than that of non-AM plants at each rate of Cd application. Enhanced S uptake was also observed in white clover colonized with *G. mosseae* in another study (Cooper and Tinker, 1978) (Table 3a, b).

The shoot Mn concentration of mycorrhizal and control plants showed approximately the same values in non-polluted soil, while significantly higher Mn concentration could be observed in control plants than in mycorrhizal plants at 50 and 100 mg Cd $kg^{-1}$ soil pollution (Tables 3a,b). However, the plant Mn concentration increased in both inoculated and non-inoculated hosts with increasing Cd application from 0 to 50 mg Cd $kg^{-1}$ soil. The lower Mn uptake of AM plants has been explained as the result of the lower activity of Mn-reducing microorganisms in the rhizosphere of mycorrhizal plants (Kothari et al., 1991). The present results are in agreement with this observation. Manganese (Mn), copper (Cu) and zinc (Zn) have low mobility in soils. Liu et al. (2000) suggested that the higher absorption surface and the shorter distance for diffusion due to extraradical AMF hyphae may result in the higher uptake of these nutrients in mycorrhizal plants. The shoot Zn concentration of AM plants was higher in unpolluted and 50 mg Cd $kg^{-1}$ soil, while lower shoot Zn concentration was measured in AM plants than in control plants in the 100 mg Cd $kg^{-1}$ soil treatment. The shoot Cu concentration of control plants was low and was not influenced by Cd application. The shoot Cu concentration of AM plants was higher than that of control plants in the 0 and 50 mg Cd $kg^{-1}$ soil treatments. In contrast, the Cu uptake of plants inoculated with *G. mosseae*-BEG12 and -HS declined compared to that of control plants in the highest soil Cd treatment, but the differences between the Cu contents in AM and non-AM plants were not significant. The enhanced and reduced acquisition of nickel (Ni) has also been reported for AM compared to non-AM plants in polluted and unpolluted soils, respectively (Guo et al., 1996; Takács and Vörös, 2003b). Significantly higher Ni concentrations were found in non-mycorrhizal plants than in mycorrhizal plants at each rate of Cd application. The shoot Ni concentration of control plants rose with the increasing availability of Cd, while the Ni uptake of AM plants was inhibited by soil Cd content in most cases (Table 3a, b).

There are very few published results on the effects of AM fungi on molybdenum (Mo) and cobalt (Co) uptake by plants. In the present study the shoot Mo concentration increased significantly in control plants compared to AM plants with increasing soil Cd application, except after *G. mosseae*-BEG12 inoculation (Tables 3a,b). The infectivity of the *G. mosseae*-BEG12 strain was greatly reduced as a consequence of high metal pollution. The Co uptake of control plants was not influenced by the increasing soil Cd content, while the response of plants colonized with various *G. mosseae* isolates to Cd treatments showed differences in shoot Co concentration.

*Table 3a*

Macroelement content (w/w%) of non-mycorrhizal and mycorrhizal white clover (*Trifolium repens* L.) shoots inoculated with different *Glomus mosseae* strains in soils treated with cadmium at three levels

| AMF treatment | 0 mg Cd $kg^{-1}$ soil | | | 50 mg Cd $kg^{-1}$ soil | | | 100 mg Cd $kg^{-1}$ soil | | |
|---|---|---|---|---|---|---|---|---|---|
| | P | N | K | P | N | K | P | N | K |
| Control | 0.06 | 1.84 | 1.37 | 0.06 | 2.21 | 1.61 | 0.05 | 1.8 | 1.71 |
| *G. mosseae*-BEG12 | 0.11 | 1.84 | 1.25 | 0.11 | 1.57 | 1.49 | 0.11 | 1.4 | 1.49 |
| *G. mosseae*-S | 0.11 | 2.15 | 0.82 | 0.13 | 1.97 | 0.82 | 0.15 | 1.82 | 0.88 |
| *G. mosseae*-M | 0.17 | 2.86 | 0.98 | 0.23 | 2.13 | 1.32 | 0.21 | 2.37 | 1.17 |
| *G. mosseae*-HS | 0.10 | 1.94 | 0.82 | 0.11 | 2.13 | 0.84 | 0.13 | 2.25 | 0.92 |
| $LSD_{5\%}$ | 0.05 | 0.45 | 0.17 | | | | | | |
| | Ca | Mg | S | Ca | Mg | S | Ca | Mg | S |
| Control | 2.96 | 0.33 | 0.10 | 3.07 | 0.31 | 0.14 | 3.62 | 0.34 | 0.16 |
| *G. mosseae*-BEG12 | 3.21 | 0.42 | 0.14 | 3.32 | 0.31 | 0.22 | 3.42 | 0.38 | 0.26 |
| *G. mosseae*-S | 2.92 | 0.41 | 0.18 | 2.93 | 0.42 | 0.21 | 3.26 | 0.43 | 0.30 |
| *G. mosseae*-M | 2.84 | 0.38 | 0.16 | 2.71 | 0.39 | 0.21 | 2.78 | 0.40 | 0.25 |
| *G. mosseae*-HS | 2.95 | 0.38 | 0.23 | 2.98 | 0.40 | 0.33 | 3.11 | 0.41 | 0.32 |
| $LSD_{5\%}$ | 0.45 | 0.03 | 0.06 | | | | | | |

*Table 3b*

Microelement content (mg $kg^{-1}$) of non-mycorrhizal and mycorrhizal white clover (*Trifolium repens* L.) shoots inoculated with different *Glomus mosseae* strains in soils treated with cadmium at three levels

| AMF treatment | 0 mg Cd $kg^{-1}$ soil | | | 50 mg Cd $kg^{-1}$ soil | | | 100 mg Cd $kg^{-1}$ soil | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mn | Mo | Zn | Mn | Mo | Zn | Mn | Mo | Zn |
| Control | 112 | 0.63 | 12.86 | 404 | 1.08 | 14.62 | 480 | 1.64 | 20.26 |
| *G. mosseae*-BEG12 | 127 | 0.81 | 15.37 | 297 | 1.35 | 16.31 | 260 | 1.09 | 15.33 |
| *G. mosseae*-S | 101 | 0.37 | 15.03 | 217 | 0.44 | 18.93 | 201 | 0.62 | 16.63 |
| *G. mosseae*-M | 102 | 0.35 | 13.80 | 165 | 0.36 | 15.43 | 199 | 0.56 | 14.70 |
| *G. mosseae*-HS | 102 | 0.49 | 16.13 | 185 | 0.78 | 20.87 | 168 | 0.61 | 18.93 |
| $LSD_{5\%}$ | 92 | 0.58 | 5.09 | | | | | | |
| | Cu | Co | Ni | Cu | Co | Ni | Cu | Co | Ni |
| Control | 6.17 | 0.11 | 1.54 | 5.24 | 0.11 | 3.51 | 6.32 | 0.10 | 6.75 |
| *G. mosseae*-BEG12 | 7.65 | 0.09 | 1.42 | 7.13 | 0.12 | 1.60 | 6.04 | 0.13 | 3.56 |
| *G. mosseae*-S | 10.18 | 0.22 | 1.69 | 8.15 | 0.12 | 1.77 | 7.53 | 0.06 | 1.40 |
| *G. mosseae*-M | 10.44 | 0.08 | 1.00 | 8.37 | 0.08 | 1.11 | 7.60 | 0.09 | 1.29 |
| *G. mosseae*-HS | 10.23 | 0.08 | 1.22 | 8.23 | 0.12 | 3.47 | 6.15 | 0.09 | 3.62 |
| $LSD_{5\%}$ | 1.91 | 0.12 | 2.70 | | | | | | |

Abbreviations of AMF treatments as in Table 2a, b.

## Conclusions

Functional diversity was found between four *G. mosseae* strains of different origin for infectivity and effectivity. The colonization of non-adapted *G. mosseae* strains (S, HS) and the *G. mosseae* strain (M) assumed to be Cd-

adapted were less affected by an increase in the available Cd content of the soil. However, non-adapted strains (*G. mosseae*-S, -HS) were able to considerably alleviate the phytotoxicity level of Cd in contaminated soils, while the *G. mosseae*-M strain isolated from Cd-polluted soil was the most efficient in the enhancement of important macronutrients (N, P, K). In the present work, the beneficial effect of mycorrhizal inoculation in improving nutrient uptake and plant growth could be clearly shown in unpolluted and poorly fertilized soil. However, the uptake of several macro- and microelements was higher in non-AM plants than in AM plants. The enhancement of nutrient uptake was restricted to a few essential nutrients in AM plants. Thus, the alleviation of phytotoxicity was the most important benefit of mycorrhizal symbiosis, according to the present results.

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# EFFECT OF TEMPERATURE AND RADIATION ON PHOTOSYNTHESIS PRODUCTIVITY IN CHESTNUT POPULATIONS (*Castanea sativa* Mill. cv. Judia)

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Studies on gas exchange parameters were made at different temperatures and radiation levels in seven seedling populations of chestnut cultivar Judia from different parts of the Trás-os-Montes region, Portugal. Differences were found for the optimal temperature, which was 31°C for JD7, 31.5°C for JD5, 32°C for JD2, 32.5°C for JD4, 33°C for JD3 and JD6, and 33.5°C for JD1 and the ink-resistant hybrid BRO310. At these values, rates of photosynthesis ranged between 8.7 and 13.4 mmol $CO_2$ $m^{-2}$ $s^{-1}$ for JD6 and JD7, while the light conditions allowing 90% of maximal photosynthesis varied between 650 (JD6) and 1385 (JD4) μmol $m^{-2}$ $s^{-1}$. JD1 showed the highest value of leaf water potential, –0.35 Mpa, and JD6, JD7 and BRO310 the lowest, –0.65 Mpa. JD1 also showed the second lowest stomatal conductance (93 mmol $m^{-2}$ $s^{-1}$) and transpiration rate (3.0 mmol $H_2O$ $m^{-2}$ $s^{-1}$).

In relation to the photosynthetic pigments, JD3 and JD7 were the most sun-loving clones having the highest values for the Chl*a*/*b* ratio (3.2 and 3.3, respectively), while JD1 had the lowest Chl/Car ratio (3.9). The overall results suggested that the JD3, JD1 and JD5 populations might increase heat stress tolerance in Judia.

**Key words:** gas exchange, pigments, fluorescence, heat stress, optimal temperature

**Abbreviations:** A, Net photosynthesis; $A_{100}$, maximal photosynthesis; $A_{90}$, value of photosynthesis at 90% of maximal rate; $A_{50}$, value of photosynthesis at 50% of maximal rate; Chl, chlorophyll; Chl*a*/*b*, Chlorophyll *a*/Chlorophyll *b* ratio; Chl/Car, Chlorophyll/Carotenoid ratio; $C_i$, internal $CO_2$ concentration; E, transpiration; Fv, variable fluorescence; Fm, maximal fluorescence; gs, stomatal conductance; PPFD, photosynthetic photon flux; $PPFD_{100}$, value of radiation for maximal photosynthesis; $PPFD_{90}$, value of radiation at 90% of maximal photosynthesis; $PPFD_{50}$, value of radiation at 50% of maximal photosynthesis; PSII, photosystem II; T, air temperature; $T_{100}$, temperature at maximal photosynthesis; $T_{90}$, temperature at 90% of maximal photosynthesis; $T_{50}$, temperature at 50% of maximal photosynthesis; WUE, water use efficiency; $\psi_w$, water potential

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## Introduction

Portugal is one of the most important producers of chestnuts in Europe. Most of the chestnut stands are planned for nut production either for immediate consumption or for export to Brazil, the USA and France, which are the main importers of Portuguese chestnuts (Cortizo et al., 1996).

Chestnut production in Portugal is mainly centred in the Northeast, in the region of Trás-os-Montes e Alto Douro, in which the cultivar Judia is grown in almost 80% of the orchards. Although this cultivar is not the most resistant to heat stress (Gomes-Laranjo et al., 2005) or to the ink disease (Gomes-Laranjo et al., 2004; Portela and Louzada, 2005), its use is still very important.

However, very little is known about the thermotolerance of chestnut cultivars during the vegetative cycle, particularly during the summer period, when the temperatures in Trás-os-Montes often reach over 32°C. The adverse conditions throughout this period, low water soil availability, high temperature and high irradiance, are known to be destabilising factors for normal chestnut growth, inducing a loss of plant vigour and consequently making the trees more susceptible to the ink pathogen (Gomes-Laranjo, 2001; Gomes-Laranjo et al., 2004; Dinis et al., 2006).

The overall process of photosynthesis is temperature dependent, since it is a biophysical and biochemical process, and elevated temperatures are usually regarded as one of the most important external influences affecting the overall photosynthetic capacity of intact photosynthesising tissues and the specific functions of various parts of the photosynthetic apparatus (Bukhov and Mohanty, 1999).

Photosynthesis is known to be a very heat-sensitive process and it can be completely inhibited by high temperature before other symptoms of stress are detected (Berry and Bjorkman, 1980).

Another external influence that affects the overall process of photosynthesis, especially in summer, is excess light. Excess light levels are potentially harmful to the pigments and proteins of the photosynthetic apparatus. Therefore, higher plants must adapt and acclimate to the light levels in their environment to optimize and preserve photosynthetic productivity (Demming-Adams and Adams, 1996).

The purpose of this study was to characterise the effect of temperature and PPFD radiation in the chestnut cultivar Judia, using parameters related to gas exchange, plant-water relations and photosynthetic pigment analysis.

## Materials and methods

The experiment was conducted at the University of Trás-os-Montes e Alto Douro, Vila Real, Portugal (41°19'N and 7°44'W; 450 m elevation), between the 2$^{nd}$ and 25$^{th}$ May. The study was carried out with seedlings of the chestnut (*Castanea sativa* Mill.) cultivar Judia from many regions of Trás-os-Montes (northeast Portugal). Populations were collected in Carrazedo de Montenegro, Vinhais, Vila Real, Macedo de Cavaleiros and Chaves. In terms of size, these plants

presented considerable variation, with 71, 49, 46, 67, 109, 44, 470 and 90 fruits per kg for JD1, JD2, JD3, JD4, JD5, JD6, JD7 and BRO 310, respectively.

Sowing was carried out in January 2006 in 50×30 cm trays filled with a mixture of sandy loam soil and peat (2:1, v/v) in a greenhouse.

Gas exchange was determined with an infrared gas analyser (IRGA, model LCipro+, from ADC BioScientific Ltd.) equipped with the microclimatic configuration. Two sequences of pre-established values for temperature and PPFD were programmed. In the temperature study, PPFD was stabilized at 1300 μmol $m^{-2}$ $s^{-1}$. The first step had a dwell time of 5 minutes in order to become stabilized at the ambient temperature. The second and third steps had a dwell time of 1 minute and a temperature of 22°C. All the other steps had a dwell time of 2 minutes each and the temperature was raised by 2°C in each step until it reached 38°C.

In order to study the PPFD curve, the temperature was stabilized at 30°C. All the steps had a dwell time of 1 minute, and the PPFD varied between 0 and 1800 μmol $m^{-2}$ $s^{-2}$. Both sequences were made six times on six different plants from each pot.

To obtain the values of water potential ($\Psi_w$) four fully developed plants were chosen and measurements were made on healthy leaves in a Scholander's pump (model Elle International).

The *in vivo* fluorescence was also studied. Values of Fv/Fm were obtained using a Plant Stress Meter MARK II (BioMonitor S.C.I. AB, Sweden). Four measurements were made per clone using a run time of 1 second and a light intensity of 400 μmol $m^{-2}$ $s^{-1}$. The leaves were kept in darkness for 30 minutes before the measurements were made.

For the determination of photosynthetic pigment content, four leaves were selected from the plants in each pot. Six 8 mm disc samples were punched out from each leaf, weighed and kept in 10 ml of 80% (v/v) acetone (pH=7.5) buffered with 25 mM Hepes (Porra et al., 1989) for 48 h. All the samples were shaken frequently (Gomes-Laranjo, 2001). After 48 h, when all the pigments were completely removed from the disks, the samples were centrifuged in a clinical centrifuge (MLW T54) at 2500×*g* for 5 minutes (Dai et al., 1992). Absorbances were measured using a Jasco V-530 (UV/VIS) spectrophotometer and the chlorophyll (Chl) and carotenoid (Car) pigments were quantified using the equations of Lichtenthaler (1987).

All the reactants used in the experiment were of analytical standard.

Analysis of variance (ANOVA) and the regression models were carried out using the Microsoft Excel and StatView 4.0 programs (Abacus concepts, Inc.).

## Results

In the temperature study, the temperature at which photosynthesis was maximal was determined. Figure 1 shows the effect of temperature on net photosynthesis for each of the clones studied.

The values of maximal net photosynthesis ranged from 8.7 for JD6 to 13.4 μmol $CO_2$ $m^{-2}$ $s^{-1}$ for JD7. These values of net photosynthesis were obtained at temperatures ($T_{100}$) of 31°C for JD7, 31.5°C for JD5, 32°C for JD2, 32.5°C for JD4, 33°C for JD3 and JD6, and 33.5°C for BRO 310.

In the study of radiation, the values of maximal PPFD, where the photosynthesis rate was maximal ($PPFD_{100}$), was 1000, 1300 and 1550 μmol $m^{-2}$ $s^{-1}$ for JD6, JD3 and JD7, while the maximal studied radiation level of 1600 μmol $m^{-2}$ $s^{-1}$ was assumed for JD1, JD2, JD4, JD5 and BRO 310.

The overall values of $PPFD_{100}$, $PPFD_{90}$, $PPFD_{50}$, $T_{100}$, $T_{90}$ and $T_{50}$, and their respective values of net photosynthesis were calculated (Table 1). $PPFD_{1000}$ values ranged from 1600 to 1000 mmol $CO_2$ $m^{-2}$ $s^{-1}$, while those for $PPFD_{50}$ were distributed in the interval between 200 and 600 μmol $CO_2$ $m^{-2}$ $s^{-1}$.

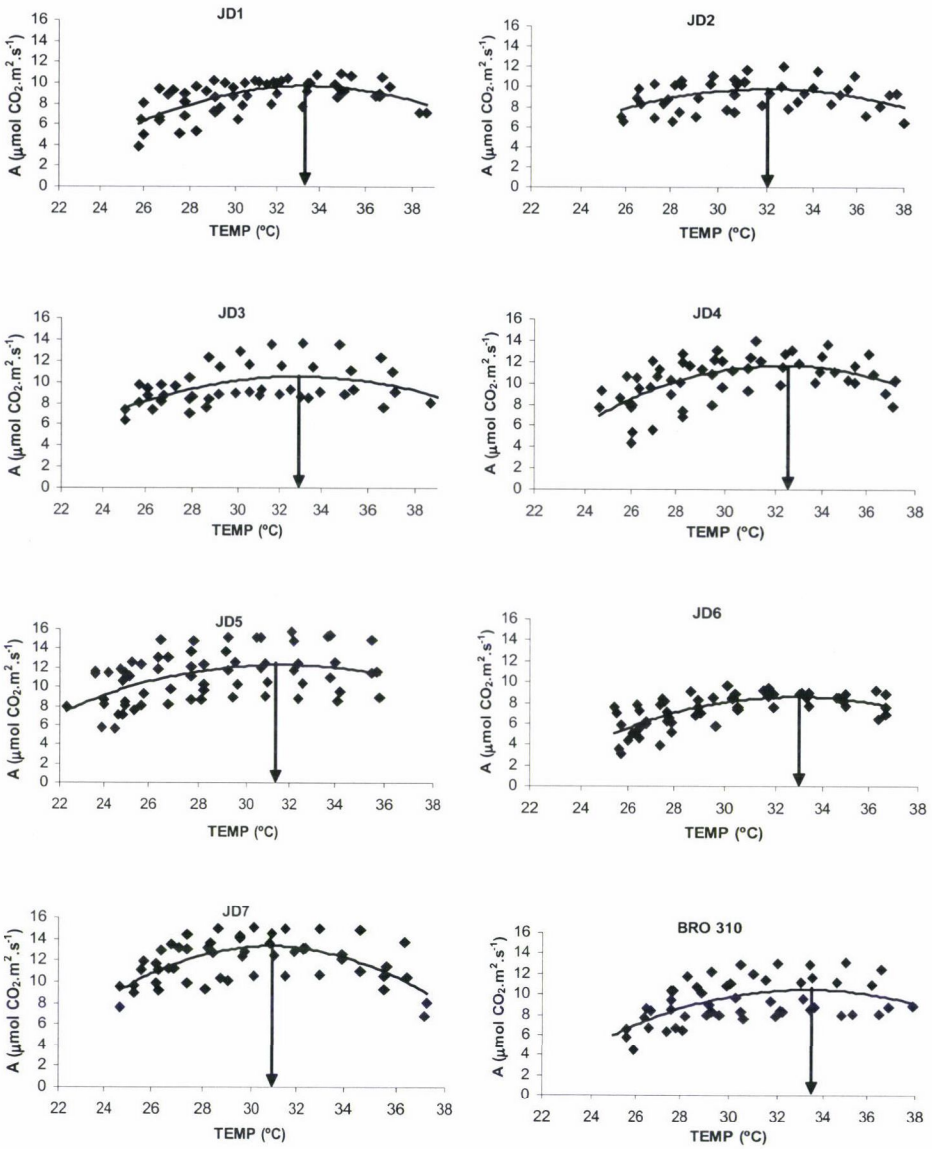



*Fig. 1.* Effect of temperature on photosynthesis of seven clones of cultivar Judia, JD1 to JD7, collected in different sites of the Trás-os-Montes region, and one hybrid, BRO 310. The polynomial equations are: JD1: $y = -0.0641x^2 + 4.2769x - 61.582$, $R^2 = 0.43$, JD2: $y = -0.052x^2 + 3.3448x - 43.972$, $R^2 = 0.17$. JD3: $y = -0.05x^2 + 3.2823x - 43.39$, $R^2 = 0.24$, JD4: $y = -0.0779x^2 + 5.047x - 70.102$, $R^2 = 0.36$, JD5: $y = -0.0546x^2 + 3.4601x - 42.519$, $R^2 = 0.22$, JD6: $y = -0.0619x^2 + 4.0879x - 58.842$, $R^2 = 0.49$, JD7: $y = -0.1105x^2 + 6.8245x - 91.98$, $R^2 = 0.42$, BRO 310: $y = -0.0642x^2 + 4.2849x - 61.048$, $R^2 = 0.28$

*Table 1*

Determination of the values of $PPFD_{100}$, $PPFD_{90}$ and $PPFD_{50}$, in μmol $CO_2$ $m^{-2}$ $s^{-1}$, and their respective rates of photosynthesis, and values (°C) of $T_{100}$, $T_{90}$ and $T_{50}$ (from polynomial equations, Fig. 1), and respective rates of photosynthesis for all clones studied

| | $PPFD_{100}$ | $A_{100}$ | $PPFD_{90}$ | $A_{90}$ | $PPFD_{50}$ | $A_{50}$ | $T_{100}$ | $A_{100}$ | $T_{90}$ | $A_{90}$ | $T_{50}$ | $A_{50}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| JD1 | 1600 | 4.90 | 1225 | 4.41 | 285 | 2.45 | 33.5 | 9.76 | 37.25 | 8.79 | 42.10 | 4.86 |
| JD2 | 1600 | 8.17 | 1315 | 7.35 | 495 | 4.08 | 32.0 | 9.81 | 36.50 | 8.84 | 41.90 | 4.88 |
| JD3 | 1300 | 6.91 | 800 | 6.21 | 375 | 3.45 | 33.0 | 10.48 | 37.40 | 9.43 | 43.05 | 5.25 |
| JD4 | 1600 | 9.16 | 1385 | 8.24 | 590 | 4.58 | 32.5 | 11.64 | 36.25 | 10.49 | 41.05 | 5.81 |
| JD5 | 1600 | 10.50 | 1365 | 9.45 | 560 | 5.22 | 31.5 | 12.30 | 36.40 | 11.09 | 42.30 | 6.15 |
| JD6 | 100 | 6.02 | 850 | 5.41 | 225 | 3.01 | 33.0 | 8.65 | 36.75 | 7.79 | 41.38 | 4.32 |
| JD7 | 1550 | 7.92 | 1050 | 7.17 | 400 | 3.95 | 31.0 | 13.39 | 34.35 | 12.06 | 38.66 | 6.70 |
| BRO310 | 1600 | 8.67 | 1320 | 7.80 | 490 | 4.34 | 33.5 | 10.45 | 37.40 | 9.41 | 42.60 | 5.22 |

PPFD polynomial equations are: JD1: $y = -6E{-}07x^2 + 0.003x + 1.6362$, $R^2 = 0.51$; JD2: $y = -1E{-}06x^2 + 0{-}0058x + 1{-}4488$, $R^2 = 0{-}71$; JD3: $y = -3E{-}06x^2 + 0.0077x + 1.9745$, $R^2 = 0.65$, JD4: $y = -3E{-}07x^2 + 0.0052x + 1.6051$, $R^2 = 0.78$; JD5: $y = -8E{-}07x^2 + 0.0068x + 1.6648$, $R^2 = 0.69$; JD6: $y = -5E{-}06x^2 + 0.01x + 1.0222$, $R^2 = 0.83$, JD7: $y = -3E{-}06x^2 + 0.0093x + 0.7171$, $R^2 = 0.89$; BRO 310: $y = -1E{-}06x^2 + 0.006x + 1.6314$, $R^2 = 0.74$ ($-6E{-}07x^2 = -6 \times 10^{-7}x^2$ etc.).

In the temperature study on the clones, a shift of 2.5°C was observed for $T_{100}$, with values ranging from 31°C to 33.5°C, while a variation of 4.4°C was observed in $T_{50}$. The most heat stress-tolerant clone was JD3, while the most susceptible was JD7. Concerning the variation between $T_{100}$ and $T_{50}$ within each clone, deviations were observed of 7.7°C for JD7, 8.4°C for JD6, 8.6°C for JD4 and JD1, 9.1°C for BRO310, 9.9°C for JD2, 10.1°C for JD3 and 10.8°C for JD5.

The correlations between $T_{100}$, photosynthesis rate and $T_{50}$ are presented in Figure 2. In this study the photosynthesis rate tended to decline as the value of $T_{100}$ rose. JD7 showed very different behaviour from that of the other clones. It exhibited the lowest values of $T_{100}$, $T_{90}$ and $T_{50}$, in addition to the highest photosynthesis rates, in contrast to JD6, which had one of the highest $T_{100}$ values but the lowest photosynthesis rate. For temperatures over that ($T_{90}$ and $T_{50}$) the values of photosynthesis clearly declined, especially in JD7, due to its rapid reduction at $T_{90}$ (34.4°C) and $T_{50}$ (38.5°C). In the other JD clones, $T_{90}$ ranged from 36.4 to 37.4°C and $T_{50}$ from 41.1 to 42.3°C. Mention should also be made of JD5, which exhibited higher photosynthesis rates at similar values of $T_{90}$ and $T_{50}$. If the highest temperature values are considered, JD1 was the clone with the best results, since its $T_{90}$ and $T_{50}$ values were 37.3 and 42.1°C, which are very close to those observed for the hybrid BRO310, which is tolerant of ink disease.

The overall analysis of temperature *versus* photosynthesis is displayed in a Euclidian distribution in Figure 3. The hybrid BRO310 showed the highest combined temperature value (39°C), which is close to that of JD1 (38.6°C) and JD3 (38.7°C). The latter had a rate of photosynthesis very similar to that of BRO310 (7.7 μmol $CO_2$ $m^{-2}$ $s^{-1}$). JD5 also exhibited some interesting characteristics, since its combined temperature was 38.1°C, but its $CO_2$ fixation rate was 9 μmol $CO_2$ $m^2$ $s^{-1}$ (17% more than the values for JD3 and BRO310).

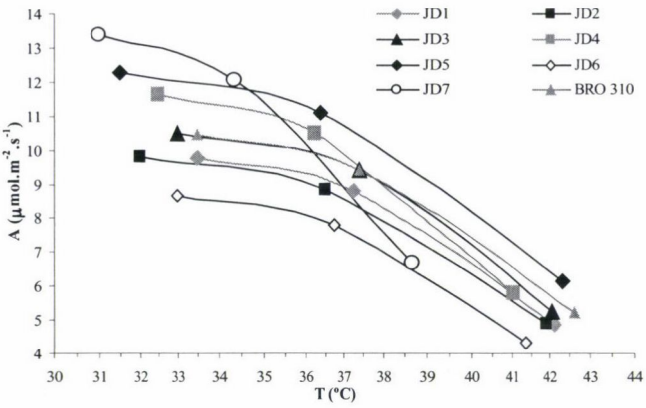



*Fig. 2.* Correlations between $T_{50}$ and $T_{100}$ (from polynomial equations, Fig. 1) for all studied clones

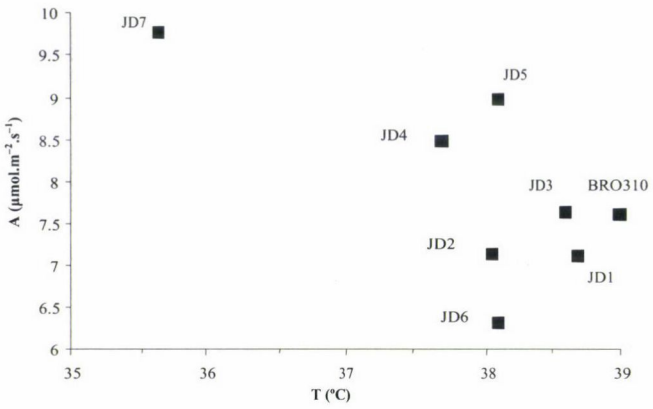



*Fig. 3.* Euclidian distribution of JD populations as a function of temperature and photosynthesis rate. Values of temperature and photosynthesis represent the weighted average of values corresponding to $T_{100}$, $T_{90}$ and $T_{50}$ (from polynomial equations, Fig. 1) multiplied by 1, 1.1 and 2, respectively

The gas exchange parameters at the optimal temperature, $T_{100}$, are presented in Table 2. The stomatal conductance rate (gs) typically decreased as $T_{100}$ increased among the clones. Clones JD5 and JD7, which had the lowest $T_{100}$ values of around 31°C, showed the highest value of gs (0.17 mmol $m^{-2}$ $s^{-1}$), while JD1 and JD6 had $T_{100}$ values of 33 and 33.5°C, associated with gs rates of 0.093 and 0.075 mmol $m^{-2}$ $s^{-1}$, respectively. The same tendency was verified for the transpiration rate, which had values of 3.8 and 2.4 mmol $m^{-2}$ $s^{-1}$ for JD4 and JD6, respectively.

The highest value of WUE was found in JD4 (3.41 µmol $CO_2$ mmol $H_2O^{-1}$), while JD3 had the lowest value of all the JD clones (2.91 µmol $CO_2$ mmol $H_2O^{-1}$), though the hybrid BRO310 had an even lower value (2.83 µmol $CO_2$ mmol $H_2O^{-1}$), representing a shift of around 0.5 µmol $CO_2$ mmol $H_2O^{-1}$ between all the JD clones. The last parameter shown is A/Ci. The highest value (0.062) was calculated for JD7 and JD4, and the smallest for JD2 (0.050).

*Table 2*
Values of gs (mmol $m^{-2}s^{-1}$), E (mmol $H_2O$ $m^{-2}s^{-1}$), WUE (μmol $CO_2$ mmol $H_2O$) and $A/C_i$ for all clones studied at $T_{100}$

| | $T_{100}$ (°C) | gs | E | WUE | A/Ci |
|---|---|---|---|---|---|
| JD1 | 33.5 | 93. | 2.97 | 3.17 | 0.055 |
| JD2 | 32.0 | 133 | 3.36 | 3.18 | 0.050 |
| JD3 | 33.0 | 106 | 3.17 | 2.91 | 0.055 |
| JD4 | 32.5 | 116 | 3.47 | 3.41 | 0.062 |
| JD5 | 31.5 | 168 | 3.76 | 3.37 | 0.061 |
| JD6 | 33.0 | 75 | 2.43 | 3.32 | 0.051 |
| JD7 | 31.0 | 166 | 3.58 | 3.31 | 0.062 |
| BRO310 | 33.5 | 126 | 3.39 | 2.83 | 0.049 |

Leaf water potential ($\Psi_w$) was also determined (Fig. 4). Among the clones, the values varied from –0.36 MPa in JD1 to –0.67 MPa in JD7 and BRO310.

The highest total chlorophyll ($Chl_{tot}$) content was found in JD4 (58.5 μg $cm^{-2}$) and the lowest in JD7 (41.7 μg $cm^{-2}$). The Chl*a*/*b* ratio ranged between 3.0 and 3.2, for JD1 and JD7, respectively. Leaf carotenoid content varied between 9.2 (JD5) and 13.4 μg $cm^{-2}$ (JD4). Variations in the Chl/Car ratio ranged from 3.9 in the highest $T_{100}$ clone, JD1, to 4.6 in the least heat-tolerant clone JD5 (Table 3).

The last parameter analysed was the Fv/Fm ratio (Fig. 5). This parameter was measured *in situ* in the morning, at an ambient temperature of 18°C, and in the afternoon at an ambient temperature of 32°C. Except for JD1, no significant differences were detected between the two measurements, but the differences between the clones were significant, JD3 having the highest Fv/Fm and JD4, JD5 and JD7 having the lowest ratio.

Cluster analysis based on temperatures $T_{100}$ and $T_{50}$ and on the corresponding rates of photosynthesis ($A_{100}$ and $A_{50}$) is displayed in Figure 6. The analysis revealed the closest association between populations JD3, JD1 and BRO310. JD4 and JD5, which constituted another group, were relatively distant from this group, while JD7 was the farthest from all the other populations.

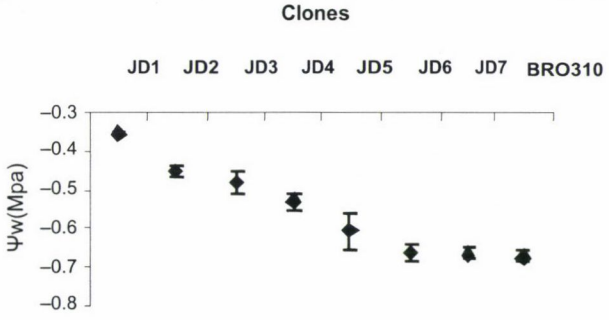



*Fig. 4.* Values of water potential for all clones studied at $T_{100}$

*Table 3*
Amounts of total chlorophyll, Chl*a*/*b* ratio, total carotenoids and Chl/Car ratio for all plants studied

| | $Chl_{tot}$ µg/cm$^2$ | $Chl_a/Chl_b$ | $Car_{tot}$ µg/cm$^2$ | Chl/Car |
|---|---|---|---|---|
| JD1 | 47.0 ± 1.8 | 3.00 ± 0.05 | 11.8 ± 0.4 | 3.92 ± 0.06 |
| JD2 | 47.5 ± 3.5 | 3.02 ± 0.04 | 11.3 ± 0.7 | 4.17 ± 0.08 |
| JD3 | 54.7 ± 4.8 | 3.18 ± 0.03 | 12.8 ± 1.1 | 4.28 ± 0.11 |
| JD4 | 58.5 ± 2.8 | 3.04 ± 0.03 | 13.4 ± 06 | 4.35 ± 0.07 |
| JD5 | 42.1 ± 3.8 | 3.12 ± 0.02 | 9.2 ± 0.8 | 4.16 ± 0.03 |
| JD6 | 50.3 ± 2.2 | 3.03 ± 0.06 | 12.1 ± 0.6 | 4.16 ± 0.03 |
| JD7 | 41.7 ± 2.5 | 3.24 ± 0.06 | 10.5 ± 0.6 | 3.99 ± 0.04 |
| BRO310 | 43.1 ± 2.9 | 2.92 ± 0.05 | 9.6 ± 0.8 | 4.52 ± 0.16 |

Values are means±SE (n=4)

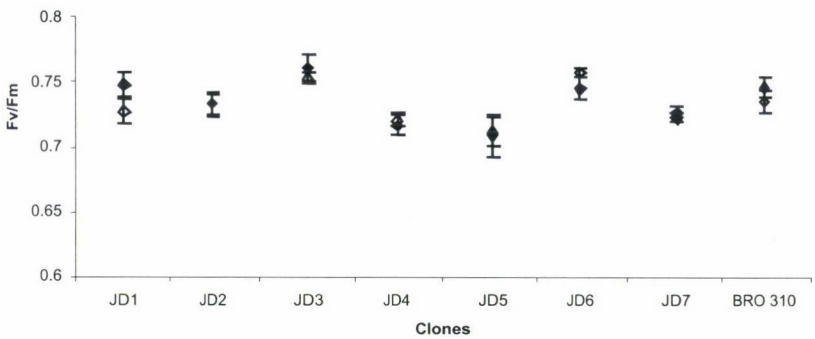



*Fig. 5.* Study of Fv/Fm ratio for all studied clones measured during the morning (black squares) and the afternoon (white squares). Ambient temperature was 18°C in the morning and 32°C in the afternoon. Bars represent the standard error (n=6)

## Discussion

These results suggest that significant differences can be found at the level of gas exchange parameters between JD populations from different regions of Trás-os-Montes.

Differences were found in the optimal temperature, which ranged between 31 and 33.5°C, and in the radiation level for 90% of maximal photosynthesis (determined at 30°C), which varied between 650 and 1320 µmol $m^{-2}$ $s^{-1}$.

Compared with previous studies (Gomes-Laranjo et al., 2005), these values of $T_{100}$ for JD are about 8°C higher, meaning that the typical value is about 24°C. The same was also true for $T_{50}$, where typical values in adult plants are around 33°C. A similar tendency occurred with the photosynthesis rate, which was much higher than those reported. According to Dinis et al. (2006), the maximal photosynthesis rate in adult plants at $T_{100}$ was 9.3, while in the seedlings studied here, the values were typically over 10.0 µmol $CO_2$ $m^2$ $s^{-1}$, the maximum being 13.4 µmol $CO_2$ $m^2$ $s^{-1}$, which was measured in JD7, representing an increase of 43%. The young age of the plants (6 months old)

might be one of the reasons for these differences. Katny et al. (2005) reported that at ambient $CO_2$ the photosynthesis rate decreased in potato plants with an increase in leaf age, being about 40% higher in young leaves than in old leaves.

High temperatures are usually regarded as one of the most important external influences affecting both the overall photosynthetic capacity of intact photosynthesising tissues, and the specific functions of various parts of the photosynthetic apparatus (Bukhov and Mohanty, 1999). The stability of many cellular membranes is important during high-temperature stress. According to Taíz and Zeiger (2002), the excessive fluidity of membrane lipids at high temperatures is correlated with loss of physiological function.

According to Figures 2 and 3, two clones might be considered as the most heat stress-tolerant. These are JD1 and JD3, which exhibited similar gas exchange parameters to the ink disease hybrid clone BRO310 (*C. crenata* × *C. sativa*). Mention should also be made of JD5, which was the most photosynthetically productive at $T_{90}$ and $T_{50}$ (17% higher than JD3), though its $T_{90}$ and $T_{50}$ values were 1°C lower than those of JD3 and JD1. In contrast, JD7 showed the worst tolerance of heat stress. These similarities and differences were confirmed by cluster analysis (Fig. 6).

JD1 exhibited the lowest stomatal conductance and transpiration rate, leading to higher leaf water potential than that observed for JD3. At $T_{100}$, both these clones showed similar photosynthesis rates, though JD1 had significantly lower $Chl_{tot}$, which could imply lower levels of soil nitrogen content and of mineral nutrient related to chlorophyll synthesis. Additionally, JD1 also had lower values of Chl*a*/*b* and Chl/Car than JD3. Under the conditions of this study, 90% of maximal photosynthesis was obtained at 1225 μmol $m^{-2}$ $s^{-1}$ in JD1, while in JD3 this value was only 800 μmol $m^{-2}$ $s^{-1}$, suggesting that JD1 could be a more sun-loving clone than JD3, which is better adapted to shady soils. Since the sunniest soils are generally hotter than the shadiest places, the overall data make physiological sense, indicating that JD1 is the most heat stress-tolerant, as reported by Boardman (1977), who suggested that high levels of sunlight induce adaptations in plants in order to maximise their photosynthetic efficiency.

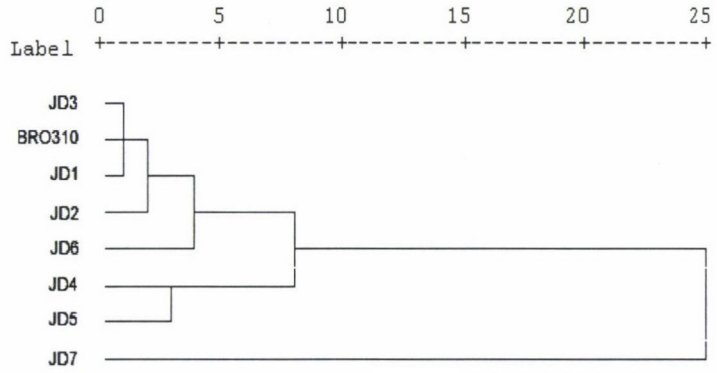



*Fig. 6.* Cluster analysis for JD populations and hybrid BRO310. Principal factors were $T_{100}$, $A_{100}$, $T_{50}$ and $A_{50}$

Light energy can be absorbed by leaves via both photochemical and non-photochemical processes. With increasing light intensity, the proportion of excess light energy increases. Excess light energy may cause photo-inhibition and photo-damage (Zhang et al., 2007). JD5 and JD7, like JD4, have low values of Fv/Fm, suggesting poor acclimation to high light (Maxwell and Johnson, 2000) and implying disturbances in the PSII reaction centre function and an increase in the level of photo-inhibition for these clones (Zhang et al., 2007).

In conclusion, variations in photosynthetic characteristics related to temperature and irradiance resistance in these clones of Judia confirm the existence of variability in this cultivar. Among the Judia populations studied, JD3 can be considered to be the population best adapted to high temperature regimes like those occurring in the Trás-os-Montes region in the summer, followed by JD1 and JD5, while JD6, JD2, JD4 and JD7 were less suitable for this region.

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# EFFECT OF INTEGRATED NUTRIENT MANAGEMENT IN A SOYBEAN–WHEAT CROP SEQUENCE ON THE YIELD, MICRONUTRIENT UPTAKE AND POST-HARVEST AVAILABILITY OF MICRONUTRIENTS ON TYPIC USTOCHREPTS SOIL

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A field experiment using various levels of phosphorus, farmyard manure (FYM) and biofertilizers was undertaken at Udaipur during 2001–02 and 2002–03 to observe the direct effect of the treatments on the yield and micronutrient uptake of soybean and their residual effect on the yield and micronutrient utilization of a subsequent wheat crop. The results revealed that the seed/grain yield of soybean and wheat significantly increased with the application of increasing levels of P, FYM and biofertilizers. The integrated use of P, FYM and biofertilizers was able to replace 20 kg $P_2O_5$ $ha^{-1}$ to soybean and 10 kg $P_2O_5$ $ha^{-1}$ to wheat.

The application of increasing levels of P, FYM and biofertilizers significantly enhanced the uptake of Zn, Cu, Mn and Fe by soybean and wheat. Among the biofertilizers, dual inoculation with phosphate-solubilising bacteria (PSB) + vesicular arbuscular mycorrhiza (VAM) gave the best performance. The integrated use of P levels and biofertilizers significantly improved the Zn and Fe uptake of soybean. The highest Zn and Fe uptake (125.77 mg $kg^{-1}$ and 562.03 mg $kg^{-1}$, respectively) was recorded with the combined use of 40 kg $P_2O_5$ $ha^{-1}$ + dual inoculation. The application of FYM and P levels resulted in a significant improvement in the organic carbon, CEC, available N and P, porosity and hydraulic conductivity of the soil after the wheat harvest. Among the biofertilizers, dual inoculation led to maximum soil-available P. Phosphorus application gave a significant decrease in the DTPA-extractable Zn and Fe content of the soil after the harvest of wheat. FYM application significantly enhanced the DTPA-extractable Zn, Cu, Mn and Fe contents of the soil.

**Key words:** P levels, FYM, biofertilizers, dual inoculation, micronutrient uptake

## Introduction

Modern-day intensive crop cultivation requires the use of chemical fertilizers, which are not only in short supply but also expensive. Therefore, the current trend is to explore the possibility of supplementing chemical fertilizers

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with organic ones, more particularly biofertilizers of microbial origin. The favourable response of vesicular arbuscular mycorrhiza (VAM) has been observed by many workers. However, few attempts have been made to evaluate the response of this phosphatic biofertilizer.

Organic manures act in many ways, such as augmenting crop growth and soil productivity. The direct effect of organics relates to the uptake of humic substances or their decomposition products, which favourably affects the growth and metabolism of plants. Indirectly, they augment the beneficial soil microorganisms and their activities and thus increase the availability of macro- and micronutrients to plants.

Integrated Nutrient Management (INM) holds out great promise for meeting the growing nutrient demands of intensive agriculture and maintaining crop productivity at higher levels with an overall improvement in the quality of the resource base. The objectives of INM are to ensure the efficient and judicious use of all the major sources of plant nutrients in an integrated manner, so as to obtain maximum economic yield without diminishing soil fertility in order to sustain agricultural productivity and farm profitability.

Fertilizers, organic manures, leguminous crop residues/wastes and biofertilizers are the main components of INM. Farmyard manure (FYM), one component of INM, favourably affects the physical, chemical and biological environments. It is well known that neither organic manure alone nor the exclusive application of chemical fertilizers can achieve yield sustainability at an optimum level under modern farming conditions, where the nutrient turnover in the soil–plant system is quite high. Long-term fertilizer trials have clearly shown the positive role of organic sources in conjunction with chemical fertilizer in maintaining the productivity of the soil by enhancing soil fertility and improving physical properties (El-Fayoumy and Ramadan, 2002; Granstedt and Kjellenberg, 1997; Behera and Ram, 2004). The use of biofertilizers not only supplements nutrients but also improves the efficiency of the nutrients applied (Somani, 2005).

## Materials and methods

A field experiment was conducted during 2001–02 and 2002–03 at Rajasthan College of Agriculture, Udaipur. The zone has a typical subtropical, sub-humid climate with mild winters, moderate summers and high humidity between July and September. The soil of the experimental field was clay loam in texture and alkaline in reaction (pH 7.8), with a bulk density of 1.54 t $m^{-3}$ and a CEC value of 20.21 cmol ($P^{+}$) $kg^{-1}$. The soil had a medium content of organic carbon (0.70 %), available N (270.4 kg $ha^{-1}$) and $P_2O_5$ (17.5 kg $ha^{-1}$) and a high content of available K (372.8 kg $ha^{-1}$). The DTPA-extractable metallic cations, Zn, Cu, Mn and Fe in the soil were 2.51, 2.18, 11.45 and 9.69 mg $kg^{-1}$, respectively.

A total of 32 treatment combinations comprising four levels of P (0, 20, 30 and 40 kg $P_2O_5$ $ha^{-1}$), two levels of FYM (0 and 10 t $ha^{-1}$) and four biofertilizers [no inoculation, phosphate-solubilizing bacteria (PSB), vesicular arbuscular mycorrhiza (VAM) and PSB + VAM] were replicated thrice in a split plot design. PSB consisted of seed inoculation with *Bacillus*

*megatherium* var. phosphaticum, while VAM involved soil application of *Glomus fasciculatum.* Soybean var. JS-335 was taken as a test crop to observe the direct effect of the treatments and wheat var. Raj 3765 was taken to observe the residual effect of the treatments. The uniform application of 30 kg N $ha^{-1}$ was common to all the treatments for soybean, along with seed inoculation with *Rhizobium,* while the recommended dose of N (90 kg $ha^{-1}$) was used for wheat.

## Results and discussion

### *Crop yield*

The application of phosphorus FYM and biofertilizers to soybean significantly increased the seed yield, while their residual effect significantly enhanced the grain yield of the subsequent wheat crop (Table 1). Among the biofertilizers, dual inoculation with PSB + VAM was found to be significantly better than either alone. The greater yield response due to dual inoculation with VAM fungus and PSB compared with PSB alone can be attributed to the activity of the VAM fungus in transporting extra phosphorus, together with the micronutrients solubilized by PSB, from the root zone and beyond into the plant roots, since in the absence of VAM hyphae these become refixed by soil constituents during the course of slower diffusion towards the plant roots. These results are in accordance with the findings of Tomar et al. (2001), El-Fayoumi and Ramadan, (2002), Potdukhe and Guldekar (2003), El-Ghandour and Galal (2002), Koreish et al (2004) and Igual et al. (2001). The interaction of phosphorus levels, FYM and biofertilizers was found to be significant in increasing the seed yield of soybean (Table 2). The seed yield obtained with dual inoculation (PSB + VAM) was significantly superior to that recorded with 20 kg $P_2O_5$ $ha^{-1}$ application. Similarly, the yield recorded with the combined use of dual inoculation + 20 kg $P_2O_5$ $ha^{-1}$ was significantly higher than that obtained with 30 kg $P_2O_5$ $ha^{-1}$, but was at par with the yield obtained in the treatment receiving 40 kg $P_2O_5$ $ha^{-1}$. The combined use of 40 kg $P_2O_5$ $ha^{-1}$ + 10 t FYM $ha^{-1}$ along with dual inoculation gave the highest seed yield, which was at par with that recorded with the combined use of 30 kg $P_2O_5$ $ha^{-1}$ + 10 t FYM $ha^{-1}$ along with dual inoculation. These effects can be attributed to the increased nutrient content in the soil followed by increased nutrient uptake by plants due to the greater availability of the nutrients from organic, inorganic and biological sources. This can be attributed to the enhanced cambial activity of the root hairs, root proliferation and cell development, leading to increased root surface areas resulting in higher growth and yield. Similar findings were also reported by Buciene et al. (2003) and Granstedt and Kjellenberg (1997).

### *Micronutrient uptake*

Increasing levels of P application and FYM significantly enhanced the uptake of Zn, Cu, Mn and Fe by soybean (Table 3) and subsequent wheat (Table 4). The increased uptake due to P fertilization can be ascribed to higher root growth and the high CEC of the soil after P application. Higher CEC in the

*Table 1*
Effect of phosphorus levels, FYM and biofertilizers on seed/grain yield (q $ha^{-1}$) of soybean and subsequent wheat

| Treatments | Seed yield of soybean | | Grain yield of wheat | |
|---|---|---|---|---|
| | 2001 | 2002 | 2001 | 2002 |
| P levels (kg $P_2O_5$ $ha^{-1}$): | | | | |
| 0 | 12.23 | 14.81 | 34.53 | 44.78 |
| 20 | 16.31 | 19.01 | 42.41 | 52.75 |
| 30 | 19.02 | 21.92 | 47.73 | 58.08 |
| 40 | 22.48 | 24.95 | 52.57 | 63.06 |
| SEm± | 0.14 | 0.16 | 0.456 | 0.432 |
| CD at 5% | 0.44 | 0.48 | 1.383 | 1.309 |
| FYM (t $ha^{-1}$): | | | | |
| 0 | 14.85 | 17.46 | 39.37 | 49.61 |
| 10 | 20.18 | 22.89 | 49.25 | 59.72 |
| SEm± | 0.10 | 0.11 | 0.322 | 0.305 |
| CD at 5% | 0.31 | 0.34 | 0.978 | 0.926 |
| Biofertilizers: | | | | |
| No inoculation | 15.05 | 17.74 | 39.76 | 50.02 |
| PSB | 17.18 | 19.90 | 44.33 | 54.72 |
| VAM | 17.91 | 20.57 | 44.74 | 55.13 |
| PSB+ VAM | 19.90 | 22.50 | 48.41 | 58.72 |
| SEm± | 0.19 | 0.17 | 0.548 | 0.585 |
| CD at 5% | 0.56 | 0.49 | 1.573 | 1.677 |

*Table 2*
Interaction effect of FYM, P levels and biofertilizers on seed yield (q $ha^{-1}$) of soybean in 2002

| Biofertilizers | $F_0P_0$ | $F_0P_{20}$ | $F_0P_{30}$ | $F_0P_{40}$ | $F_{10}P_0$ | $F_{10}P_{20}$ | $F_{10}P_{30}$ | $F_{10}P_{40}$ |
|---|---|---|---|---|---|---|---|---|
| No inoculation | 9.33 | 13.61 | 15.00 | 18.80 | 16.25 | 19.46 | 22.00 | 26.83 |
| PSB | 12.91 | 16.02 | 18.92 | 21.77 | 16.79 | 21.22 | 23.13 | 28.44 |
| VAM | 12.69 | 16.81 | 19.44 | 22.33 | 17.25 | 22.13 | 26.59 | 27.30 |
| PSB+VAM | 14.50 | 18.96 | 22.34 | 25.30 | 18.15 | 23.90 | 27.97 | 28.87 |
| SEm± | 0.58 | | | | | | | |
| CD at 5% | 1.66 | | | | | | | |

vicinity of the roots stimulated nodulation and $N_2$ fixation, causing greater cell division and non-pectic carbohydrate deposition in the root cell walls. The increased nutrient content of the root also increased the production of root hairs and the lateral root tip development (Bear, 1964). All these attributes favoured enhanced access to and absorption of micronutrients with limited diffusivity in the soil. Similar findings were reported by El-Fayoumi and Ramadan (2002).

The increased uptake of micronutrients due to FYM application is ascribed to the role of organic matter in supplying micronutrients, along with an improvement in the physical properties of the soil. These improvements, in turn, bring the nutrients into soluble, available form, which ultimately improves the nutrient status of the plant parts. These results are in close conformity with the

*Table 3*
Effect of phosphorus levels, FYM and biofertilizers on Zn, Cu, Mn and Fe uptake (mg $kg^{-1}$) by soybean

| Treatments | 2001 | 2002 | Mean | 2001 | 2002 | Mean |
|---|---|---|---|---|---|---|
| P levels (kg $P_2O_5$ $ha^{-1}$): | | Zn uptake | | | Cu uptake | |
| 0 | 79.01 | 76.14 | 77.57 | 106.38 | 119.97 | 113.17 |
| 20 | 98.23 | 92.94 | 95.58 | 140.77 | 151.80 | 146.28 |
| 30 | 111.30 | 102.86 | 107.08 | 162.60 | 172.26 | 334.86 |
| 40 | 128.55 | 115.79 | 122.17 | 190.94 | 198.22 | 194.58 |
| SEm± | 0.80 | 1.16 | | 3.27 | 3.37 | |
| CD at 5% | 2.43 | 3.52 | | 9.93 | 10.21 | |
| FYM (t $ha^{-1}$): | | | | | | |
| 0 | 84.85 | 80.92 | 82.88 | 123.64 | 135.23 | 129.43 |
| 10 | 123.70 | 112.94 | 118.32 | 176.71 | 185.90 | 181.30 |
| SEm± | 0.57 | 0.82 | | 2.31 | 2.38 | |
| CD at 5% | 1.72 | 2.49 | | 7.02 | 7.22 | |
| Biofertilizers: | | | | | | |
| No inoculation | 89.30 | 84.41 | 86.85 | 129.10 | 146.67 | 137.88 |
| PSB | 101.82 | 94.85 | 98.33 | 147.20 | 157.90 | 152.55 |
| VAM | 107.00 | 99.34 | 103.17 | 153.76 | 163.99 | 158.57 |
| PSB+ VAM | 118.96 | 109.13 | 114.04 | 170.64 | 179.68 | 175.16 |
| SEm± | 1.53 | 1.57 | | 3.19 | 3.69 | |
| CD at 5% | 4.38 | 4.52 | | 9.16 | 10.59 | |
| P levels (kg $P_2O_5$ $ha^{-1}$): | | Mn uptake | | | Fe uptake | |
| 0 | 140.61 | 151.13 | 145.87 | 287.03 | 325.49 | 306.26 |
| 20 | 184.57 | 188.87 | 187.72 | 375.46 | 406.52 | 390.99 |
| 30 | 211.84 | 212.12 | 211.98 | 429.39 | 455.71 | 442.55 |
| 40 | 248.69 | 243.02 | 245.85 | 500.09 | 518.88 | 509.48 |
| SEm± | 5.04 | 3.25 | | 6.18 | 4.29 | |
| CD at 5% | 15.28 | 9.86 | | 18.76 | 13.01 | |
| FYM (t $ha^{-1}$): | | | | | | |
| 0 | 162.25 | 168.99 | 165.62 | 328.38 | 360.83 | 344.60 |
| 10 | 230.61 | 228.58 | 229.59 | 467.60 | 492.47 | 480.03 |
| SEm± | 3.56 | 2.30 | | 4.37 | 3.03 | |
| CD at 5% | 10.81 | 6.97 | | 13.26 | 9.20 | |
| Biofertilizers: | | | | | | |
| No inoculation | 168.82 | 174.04 | 171.43 | 342.41 | 374.13 | 328.27 |
| PSB | 192.49 | 195.36 | 193.92 | 390.45 | 419.65 | 405.05 |
| VAM | 201.05 | 202.96 | 202.01 | 407.08 | 435.36 | 421.22 |
| PSB+ VAM | 223.35 | 222.79 | 223.07 | 452.03 | 477.46 | 464.74 |
| SEm± | 4.17 | 3.53 | | 5.59 | 6.69 | |
| CD at 5% | 11.96 | 10.12 | | 16.03 | 19.19 | |

findings of Tarafdar and Rao (2001). Inoculation with PSB and VAM also increased the uptake of micronutrients by soybean (Table 3) and subsequent wheat (Table 4). The observed increase could be attributed to the beneficial effect observed in terms of improved nutrition, growth and root mass, on account of the growth-promoting substances released by PSB and VAM, besides the better utilization of available nutrients (Morgan et al., 2001). These results corroborate the findings of Rani and Sanoria (2000) and Shalaby and Hanna (1998).

*Table 4*
Residual effect of phosphorus levels, FYM and biofertilizers on Zn, Cu, Mn and Fe uptake (mg $kg^{-1}$) by wheat

| Treatments | 2001–02 | 2002–03 | Mean | 2001–02 | 2002–03 | Mean |
|---|---|---|---|---|---|---|
| P levels (kg $P_2O_5$ $ha^{-1}$): | | Zn uptake | | | Cu uptake | |
| 0 | 167.40 | 209.38 | 188.39 | 137.33 | 169.86 | 153.74 |
| 20 | 198.73 | 236.22 | 217.47 | 167.03 | 197.57 | 182.30 |
| 30 | 218.82 | 252.18 | 235.50 | 187.47 | 215.88 | 201.67 |
| 40 | 236.07 | 266.07 | 251.07 | 205.67 | 232.65 | 219.16 |
| SEm± | 2.39 | 3.33 | | 2.26 | 2.57 | |
| CD at 5% | 7.26 | 10.09 | | 6.84 | 7.79 | |
| FYM (t $ha^{-1}$): | | | | | | |
| 0 | 177.88 | 214.57 | 196.22 | 151.05 | 180.02 | 165.53 |
| 10 | 232.63 | 267.35 | 249.99 | 197.70 | 227.96 | 212.83 |
| SEm± | 1.69 | 2.35 | | 1.59 | 1.82 | |
| CD at 5% | 5.13 | 7.14 | | 4.84 | 5.51 | |
| Biofertilizers: | | | | | | |
| No inoculation | 183.91 | 220.10 | 202.00 | 156.26 | 186.40 | 171.33 |
| PSB | 204.90 | 240.68 | 222.79 | 174.31 | 203.99 | 189.15 |
| VAM | 207.55 | 243.39 | 225.47 | 176.10 | 205.79 | 190.94 |
| PSB+ VAM | 224.66 | 259.68 | 242.17 | 190.83 | 219.79 | 205.31 |
| SEm± | 3.56 | 5.71 | | 2.67 | 3.38 | |
| CD at 5% | 10.20 | 16.39 | | 7.65 | 9.71 | |
| P levels (kg $P_2O_5$ $ha^{-1}$): | | Mn uptake | | | Fe uptake | |
| 0 | 196.45 | 275.59 | 236.02 | 943.36 | 1281.63 | 1112.49 |
| 20 | 238.72 | 320.03 | 279.37 | 1144.51 | 1479.45 | 1311.98 |
| 30 | 267.18 | 347.93 | 307.55 | 1279.95 | 1601.73 | 1440.84 |
| 40 | 292.73 | 372.49 | 332.61 | 1397.76 | 1706.07 | 1551.91 |
| SEm± | 3.42 | 5.57 | | 13.17 | 24.36 | |
| CD at 5% | 10.39 | 16.90 | | 39.95 | 73.90 | |
| FYM (t $ha^{-1}$): | | | | | | |
| 0 | 217.28 | 294.47 | 255.87 | 1031.39 | 1353.78 | 1192.58 |
| 10 | 280.26 | 363.55 | 321.90 | 1351.40 | 1680.66 | 1516.03 |
| SEm± | 2.42 | 3.94 | | 9.31 | 17.23 | |
| CD at 5% | 7.35 | 11.95 | | 28.25 | 52.26 | |
| Biofertilizers: | | | | | | |
| No inoculation | 222.93 | 300.67 | 261.80 | 1069.09 | 1388.40 | 1228.74 |
| PSB | 248.65 | 329.03 | 288.84 | 1192.06 | 1518.95 | 1355.50 |
| VAM | 251.25 | 331.91 | 291.58 | 1202.33 | 1529.01 | 1365.67 |
| PSB+ VAM | 272.26 | 354.43 | 313.34 | 1302.09 | 1632.51 | 1467.30 |
| SEm± | 3.77 | 5.28 | | 18.02 | 23.77 | |
| CD at 5% | 10.80 | 15.15 | | 51.69 | 68.18 | |

The uptake of Zn (Table 5) and Fe (Table 6) by soybean increased significantly with the combined use of P and biofertilizers. The microbial strains used as inoculants derive their nutrition from the soil or the host plant, so their growth depends on the nutrition provided by the soil, especially in the root zone. An increase in macronutrients would help in the proliferation of soil microflora in general, which, in turn, influence the rate of mineralization, facilitating the increased availability and uptake of essential nutrients, including micronutrients

(Senthil Kumar and Arockiasamy, 1995). VAM fungi have been reported to increase nutrient uptake by shortening the distance that nutrients must diffuse through soil into the root (Hayman, 1975), thus accelerating the rate of nutrient absorption and their concentration at the absorbing surface (Barea et al., 2002). Dual inoculation with PSB + VAM helps in the development of an extensive root system, a higher percentage of mycorrhizal infection and improved nodulation (Somani, 2002; Rabie and Humiany, 2004). These factors ensured the increased availability of water and nutrients to the plants. The results of the present investigation are in line with the earlier findings of Totawat et al. (2000) and Mankarios et al. (1995).

*Table 5*
Interaction effect of P levels and biofertilizers on Zn uptake (mg $kg^{-1}$) by soybean in 2002

| Biofertilizers | P levels (kg $P_2O_5$ $ha^{-1}$) | | | |
|---|---|---|---|---|
| | 0 | 20 | 30 | 40 |
| No inoculation | 70.44 | 78.84 | 82.74 | 105.63 |
| PSB | 74.45 | 90.90 | 98.30 | 115.77 |
| VAM | 75.46 | 95.85 | 110.05 | 116.00 |
| PSB+VAM | 84.20 | 106.18 | 120.36 | 125.77 |
| SEm± | 3.15 | | | |
| CD at 5% | 9.03 | | | |

*Table 6*
Interaction effect of P levels and biofertilizers on Fe uptake (mg $kg^{-1}$) by soybean in 2002

| Biofertilizers | P levels (kg $P_2O_5$ $ha^{-1}$) | | | |
|---|---|---|---|---|
| | 0 | 20 | 30 | 40 |
| No inoculation | 303.70 | 348.71 | 369.00 | 475.12 |
| PSB | 319.69 | 400.11 | 438.35 | 520.46 |
| VAM | 321.01 | 416.82 | 485.69 | 517.90 |
| PSB+VAM | 357.56 | 460.42 | 529.81 | 562.03 |
| SEm± | 13.38 | | | |
| CD at 5% | 38.37 | | | |

*Post-harvest soil properties and micronutrient status*

The application of phosphorus resulted in a significant improvement in the organic carbon, CEC and available N and P status of the soil after the harvest of wheat (Table 7). The observed increase in organic carbon and CEC may be due to enhanced root growth, which then becomes part of the soil organic matter, as also reported by Prasad (1983) and Prakash et al. (2002). The significant build-up of soil-available N could be ascribed to the increased activity of rhizobia, resulting in the greater accumulation of N in the soil. The P status of the soil improved with increasing levels of P application due to the only partial recovery of applied P by the crops. Biofertilization with exotic strains of PSB also contributed to the increased solubility of unavailable native soil phosphate, while the VAM hyphae extracted P from deeper layers (Barea et al., 2002).

*Table 7*
Effect of phosphorus levels, FYM and biofertilizers on some soil properties after harvest of wheat

| Treatments | 2001–02 | 2002–03 | 2001–02 | 2002–03 | 2001–02 | 2002–03 |
|---|---|---|---|---|---|---|
| P levels (kg $P_2O_5$ $ha^{-1}$): | Organic carbon (%) | | CEC [cmol ($P^+$) $kg^{-1}$] | | N (kg / ha) | |
| 0 | 0.66 | 0.67 | 20.15 | 20.28 | 272.58 | 268.32 |
| 20 | 0.75 | 0.78 | 21.17 | 21.59 | 281.38 | 276.99 |
| 30 | 0.80 | 0.82 | 21.90 | 22.36 | 287.12 | 282.77 |
| 40 | 0.85 | 0.90 | 22.18 | 22.83 | 290.20 | 285.57 |
| SEm± | 0.013 | 0.031 | 0.151 | 0.483 | 4.16 | 2.30 |
| CD at 5% | 0.040 | 0.094 | 0.460 | 1.465 | 12.62 | 6.97 |
| FYM (t $ha^{-1}$): | | | | | | |
| 0 | 0.67 | 0.70 | 19.79 | 20.22 | 268.91 | 264.69 |
| 10 | 0.87 | 0.89 | 22.91 | 23.31 | 296.72 | 292.13 |
| SEm± | 0.009 | 0.022 | 0.107 | 0.341 | 2.94 | 1.63 |
| CD at 5% | 0.028 | 0.066 | 0.325 | 1.036 | 8.92 | 4.93 |
| Biofertilizers: | | | | | | |
| No inoculation | 0.74 | 0.77 | 21.15 | 21.57 | 281.73 | 275.00 |
| PSB | 0.76 | 0.79 | 21.28 | 21.66 | 282.69 | 278.18 |
| VAM | 0.77 | 0.80 | 21.45 | 21.89 | 282.92 | 278.41 |
| PSB+ VAM | 0.79 | 0.82 | 21.53 | 21.94 | 282.94 | 282.65 |
| SEm± | 0.014 | 0.020 | 0.143 | 0.287 | 5.17 | 2.32 |
| CD at 5% | NS | NS | NS | NS | NS | NS |
| P levels (kg $P_2O_5$ $ha^{-1}$): | $P_2O_5$ (kg/ha) | | Porosity (%) | | Hydraulic conduct.(cm $h^{-1}$) | |
| 0 | 14.05 | 13.99 | 44.71 | 44.82 | 0.33 | 0.34 |
| 20 | 18.26 | 18.68 | 45.34 | 45.46 | 0.33 | 0.37 |
| 30 | 20.36 | 18.87 | 45.40 | 45.55 | 0.34 | 0.38 |
| 40 | 21.50 | 20.02 | 45.30 | 45.63 | 0.37 | 0.39 |
| SEm± | 0.17 | 0.23 | 1.36 | 0.54 | 0.012 | 0.011 |
| CD at 5% | 0.52 | 0.69 | NS | NS | NS | NS |
| FYM (t $ha^{-1}$): | | | | | | |
| 0 | 17.80 | 17.11 | 43.83 | 44.02 | 0.31 | 0.32 |
| 10 | 19.29 | 18.68 | 46.54 | 46.71 | 0.38 | 0.42 |
| SEm± | 0.12 | 0.16 | 0.96 | 0.38 | 0.008 | 0.008 |
| CD at 5% | 0.37 | 0.49 | NS | 1.16 | 0.026 | 0.024 |
| Biofertilizers: | | | | | | |
| No inoculation | 17.75 | 16.75 | 44.97 | 45.28 | 0.34 | 0.36 |
| PSB | 18.24 | 17.71 | 45.18 | 45.31 | 0.35 | 0.37 |
| VAM | 18.71 | 18.18 | 45.28 | 45.43 | 0.34 | 0.37 |
| PSB+ VAM | 19.47 | 18.93 | 45.31 | 45.44 | 0.34 | 0.37 |
| SEm± | 0.21 | 0.28 | 1.01 | 0.37 | 0.014 | 0.013 |
| CD at 5% | 0.62 | 0.79 | NS | NS | NS | NS |

NS=non-significant

The micronutrient (zinc and iron) content in the soil decreased significantly after the wheat harvest with increasing P levels (Table 8). The decrease in the post-harvest availability of Zn can be attributed to the phenomenon of P/Zn antagonism, as reported by Haldar and Mandal (1981) and Davis and Rhoads (1994). As the soil was alkaline (pH 7.8), the precipitation of Zn as $Zn(OH)_2$ or $ZnCO_3$ would have occurred, besides the formation of insoluble $Zn_3(PO_4)_2$ compounds (Brown et al., 1960). The decrease in the DTPA-extractable Fe content of the soil due to P application is attributable to the reaction of P with iron oxides and hydroxides, leading to fixation and a decrease in the available Fe status. Similar results were reported by Laxmi and Narayanan (1981).

*Table 8*
Residual effect of phosphorus levels, FYM and biofertilizers on DTPA-extractable zinc, copper, manganese and iron (mg $kg^{-1}$) of surface soil (0–15 cm) after harvest of wheat

| Treatments | 2001–02 | 2002–03 | Mean | 2001–02 | 2002–03 | Mean |
|---|---|---|---|---|---|---|
| P levels (kg $P_2O_5$ $ha^{-1}$): | | Zinc | | | Copper | |
| 0 | 2.17 | 2.07 | 2.12 | 1.99 | 2.02 | 2.00 |
| 20 | 2.02 | 1.93 | 1.97 | 1.98 | 2.03 | 2.00 |
| 30 | 1.95 | 1.86 | 1.90 | 1.96 | 2.01 | 1.98 |
| 40 | 1.88 | 1.78 | 1.83 | 1.95 | 1.98 | 1.96 |
| SEm± | 0.015 | 0.021 | | 0.017 | 0.035 | |
| CD at 5% | 0.047 | 0.063 | | NS | NS | |
| FYM (t $ha^{-1}$): | | | | | | |
| 0 | 1.95 | 1.74 | 1.84 | 1.93 | 1.97 | 1.95 |
| 10 | 2.06 | 2.07 | 2.06 | 2.01 | 2.05 | 2.03 |
| SEm± | 0.011 | 0.015 | | 0.012 | 0.025 | |
| CD at 5% | 0.033 | 0.044 | | 0.037 | 0.075 | |
| Biofertilizers: | | | | | | |
| No inoculation | 1.98 | 1.88 | 1.93 | 1.96 | 2.00 | 1.98 |
| PSB | 1.99 | 1.90 | 1.94 | 1.97 | 2.01 | 1.99 |
| VAM | 2.03 | 1.92 | 1.97 | 1.97 | 2.02 | 1.99 |
| PSB+ VAM | 2.03 | 1.93 | 1.98 | 1.97 | 2.02 | 1.99 |
| SEm± | 0.023 | 0.019 | | 0.013 | 0.027 | |
| CD at 5% | NS | NS | | NS | NS | |
| P levels (kg $P_2O_5$ $ha^{-1}$): | | Manganese | | | Iron | |
| 0 | 10.93 | 10.26 | 10.59 | 9.35 | 10.21 | 9.78 |
| 20 | 10.91 | 10.27 | 10.59 | 9.09 | 9.97 | 9.53 |
| 30 | 10.87 | 10.26 | 10.56 | 8.97 | 9.87 | 9.42 |
| 40 | 10.86 | 10.28 | 10.57 | 8.87 | 9.78 | 9.32 |
| SEm± | 0.113 | 0.048 | | 0.019 | 0.040 | |
| CD at 5% | NS | NS | | 0.057 | 0.122 | |
| FYM (t $ha^{-1}$): | | | | | | |
| 0 | 10.40 | 9.67 | 10.03 | 8.81 | 9.66 | 9.23 |
| 10 | 11.38 | 10.86 | 11.12 | 9.33 | 10.26 | 9.79 |
| SEm± | 0.079 | 0.034 | | 0.013 | 0.028 | |
| CD at 5% | 0.241 | 0.103 | | 0.040 | 0.086 | |
| Biofertilizers: | | | | | | |
| No inoculation | 10.87 | 10.25 | 10.56 | 9.05 | 9.92 | 9.48 |
| PSB | 10.89 | 10.26 | 10.57 | 9.06 | 9.95 | 9.50 |
| VAM | 10.90 | 10.27 | 10.58 | 9.08 | 9.97 | 9.52 |
| PSB+ VAM | 10.91 | 10.29 | 10.06 | 9.09 | 10.00 | 9.54 |
| SEm± | 0.163 | 0.062 | | 0.036 | 0.063 | |
| CD at 5% | NS | NS | | NS | NS | |

NS=non-significant

The incorporation of FYM before sowing soybean significantly increased soil porosity and hydraulic conductivity (Table 7). The observed increase in the hydraulic conductivity of the soil is due to the improved aggregation of the surface layer (Brar and Pasricha, 1998), while the enhanced porosity is due to a decrease in the bulk density of the soil with FYM. Similar improvements in physical properties were reported by El-Fayoumy and Ramadan (2002) and El-Sersawy et al. (1993).

The DTPA-extractable contents of Zn, Cu, Mn and Fe increased significantly after the harvest of wheat with the incorporation of FYM. Presumably FYM increased the Zn, Cu, Mn and Fe content by supplying complexing agents, which formed stable complexes with these micronutrients. Organometallic complexes reduce the adsorption, fixation and precipitation of these metals in the soil. These results are in conformity with those reported by Jalali et al. (1990), Kumar et al. (2000) and Granstedt and Kjellenberg (1997). The improvement may also be attributed to the release of native micronutrients contained in the FYM as a consequence of microbial decomposition (Jarecki, 1991).

It can be concluded from the results of this experiment that the integrated use of P, FYM, and dual inoculation with biofertilizers can reduce the fertilizer P requirements of soybean to the extent of 20 kg $P_2O_5$ $ha^{-1}$ and that of the subsequent wheat crop by 10 kg $P_2O_5$ $ha^{-1}$. It also leads to the better utilization of micronutrients by both the crops. The post-harvest availability of Zn, Cu, Mn and Fe increased following the application of FYM, but that of Zn and Fe decreased significantly with increasing levels of $P_2O_5$.

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# CHEMICAL QUALITY PARAMETERS OF MAIZE HYBRIDS IN VARIOUS FAO MATURITY GROUPS AS CORRELATED WITH YIELD AND YIELD COMPONENTS

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An experiment was set up at five locations in Hungary in 2005, in a randomised block design with four replications. At each location 24 hybrids were tested from each of four maturity groups (FAO 200, FAO 300, FAO 400, FAO 500). Evaluations were made of the yield average (t/ha) and the yield components of the sample ears: ear length, number of kernel rows, thousand-kernel mass and kernel/cob ratio. This was followed by chemical analysis to determine the protein, oil and starch contents of the kernels. The chemical quality parameters were recorded for almost 100 hybrids, and the correlations of the protein, oil and starch contents with yield and yield components were analysed. It was found that in all the maturity groups the yield was closely correlated with the thousand-kernel mass (0.72). In each maturity group the highest yield averages were associated with the greatest average starch contents, except for the FAO 500 group in the Szarvas location, where the development of secondary ears contributed to the achievement of the highest yield average. A very close correlation was found between the starch content and the thousand kernel mass (0.91). The variety caused greater differences in protein content than the location. This was also true for the oil content in the FAO 200 and FAO 400 groups, but only in the FAO 400 group in the case of starch content. More starch was incorporated at wetter locations, where the protein content of the samples was lower.

**Key words:** maize hybrid, FAO maturity group, yield, yield component, protein, oil, starch

## Introduction

In the years to come, increasing emphasis will be placed in maize breeding on the chemical analysis of maize hybrids and the improvement of chemical quality parameters (protein, oil and starch content). Among the chemical components decisive for quality, the protein, fat and starch contents can be influenced to a substantial effect by breeding (Bálint, 1977; Kralovánszky and Manninger, 1985). If the nutritional value of maize is to be improved,

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modifications will be required primarily in the protein and oil contents and in the quality of the protein. When high maize yields per unit area are achieved in the field, they generally have a relatively low protein content, while varieties containing over 12% protein tend to have lower yields (Sprague, 1955). Around 80% of the protein in the maize kernel accumulates in the endosperm, and 40% of this is zein, which does not contain the amino acids lysine or tryptophan, both of which are essential in animal nutrition (Győrffy and I'só, 1970). Improvements in both the quantity and quality of the protein are of fundamental importance whether maize is being bred for either animal feeding or human consumption (Pásztor and Győri, 1991; Mertz, 1992). In many countries maize is a staple food (Sharobeem et al., 1986). Genetic research aimed at increasing the lysine content began in the USA in the 1960s (Bates et al., 1965; Watson and Ramstadt, 1991). Data on fluctuations in protein content at different growing sites were first published in Hungary by Bálint (1977), who found differences of 2.6–4.3% between varieties and 2.4–5.5% between locations. In experiments carried out by Gyenesné-Hegyi et al. (2001), however, the difference between hybrids was greater than that between locations. Samples were found to have lower protein content in wet years (Prokszáné Paplogó et al., 1995).

Interest in the utilisation of vegetable oils is stimulated both by the desire for healthy nutrition and by the constant rise in the price of petroleum products. Due to their complex composition, vegetable oils can be used to make many types of products and are biologically decomposable. Maize oil is ideal for the production of various types of snacks, where extremely stable oils are required. Maize oil is a valuable by-product of the starch manufacturing process, and also plays an important role in animal nutrition, due to its high energy content. Maize kernels have an average oil content of 4.4%, most of which is located in the germ. An increase in the germ ratio has a favourable effect in improving the ratio of more valuable protein fractions, while also increasing the kernel oil content (Earle et al., 1946; Sprague and Brimhall, 1949). Maize with a higher oil content was found to have greater biological value than that with a low content (Schneider et al., 1952). This is due to the fact that the energy value of the oil is almost 2.5 times that of the starch. The environment has less influence on the oil content than on the protein content (Sprague and Brimhall, 1949; Gyenesné-Hegyi et al., 2001). Experiments have proved that maize growing at locations with low mean temperature have poorer oil content (Veneni, 1971).

Among the grain fodder crops grown in Hungary, maize has the highest starch value (Gundel, 1985). The kernels have a dry matter content of 89%, of which 72% is starch (Inglett, 1970). In normal, starch-type maize the starch consists of approx. 25% amylose and 75% amylopectin. In experiments carried out by Pásztor et al. (1998) there was no substantial difference in the starch content in different years, but as the thousand-kernel mass increased, there was a rise in the starch and fat contents in the hybrids included in the experiment. Bálint (1977) found that lower thousand-kernel mass was associated with a

smaller endosperm, a lower accumulation of starch and a relative increase in the kernel protein content. Interest has recently focussed on the use of biofuels, which use renewable plant biomass as raw material and which pollute the environment less than fossil fuels. Wheat, maize or maize stalks could be possible sources of raw material for bioethanol production. Maize is possibly more favourable, as less maize (2.72 t) than wheat (3.14 t) is required to produce a tonne of ethanol. A further advantage of maize is its higher yield average. In 2004 average yields were 7.1 t maize and 5.1 t wheat, so more than one and a half times as much bioethanol could be produced from unit area if maize were grown (László and Réczey, 2000; Emőd et al., 2005).

## Materials and methods

An experiment was set up at five locations in Hungary (Iregszemcse, Kaba, Martonvásár, Mezőkövesd, Szarvas) in 2005, in a randomised block design with four replications. At each location 24 hybrids were tested from each of four maturity groups (FAO 200, FAO 300, FAO 400, FAO 500). The experiments were sown and harvested mechanically. Prior to harvest, five sample ears were taken from each plot, and the yield average per hectare of each hybrid was corrected based on the ear mass of the sample ears.

Evaluations were made of the yield average (t/ha) and the yield components of the sample ears: ear length, number of kernel rows, thousand-kernel mass and kernel/cob ratio. This was followed by chemical analysis to determine the protein, oil and starch contents of the kernels. The measurements were made using a Fourier transform NIR spectrometer (Bruker, Ettlingen, Germany).

The yield data, yield components and chemical parameters were evaluated for each maturity group to determine the correlation between yield data and chemical parameters.

## Results

The yield average in the FAO 200 maturity group was 9.43 t/ha (Table 1). The significantly highest yields were obtained in Martonvásár (10.52 t/ha) and the lowest in Mezőkövesd (7.32 t/ha). This can be attributed to the rainfall distribution in 2005, when the highest rainfall during the vegetation period was recorded in Martonvásár (581.2 mm) and the lowest in Mezőkövesd (460.4 mm). There was no significant difference between the yields achieved in Iregszemcse, Kaba and Szarvas (9.88, 9.87 and 9.55 t/ha, respectively). The highest thousand-kernel mass was also recorded in Martonvásár (349.1 g), where the kernel/cob ratio was the most favourable (88:12). Although the longest ears were found in Kaba (18.93 cm), many of them did not set seeds at the tip, leading to the worst kernel/cob ratio at this location (85:15). The highest protein and oil contents were recorded in samples from Kaba (8.46 and 3.60%, respectively), together with the smallest starch accumulation (65.34%). In contrast, samples from Martonvásár, which gave the highest total yield, had the greatest starch content (68.01%) and the lowest protein and oil contents (6.98 and 3.45%, respectively).

In the FAO 300 group the highest yield averages were obtained in Martonvásár and Iregszemcse (10.84 and 10.68 t/ha, respectively) (Table 2). The high yield in Martonvásár could be attributed to the fact that both the thousand-kernel mass and the length of the main ear (18.76 cm) were greatest at this location. In close correlation with the yield average, the starch accumulation in the kernels was greatest in Martonvásár (68.25%), while the protein and oil contents were the lowest (7.35 and 3.45%, respectively). The abundant rainfall favoured the incorporation of starch, while the samples became poor in protein. The ecological conditions in Kaba were most favourable for the development of high protein and oil contents (8.40 and 3.62%, respectively), which correlated closely with the lowest starch content in the endosperm (65.57%), averaged over the maize hybrids.

In the FAO 400 group the greatest yield average was achieved in Szarvas (10.40 t/ha), but this was not significantly better than those recorded in Martonvásár (10.23 t/ha), Kaba (10.02 t/ha) and Iregszemcse (9.81 t/ha) (Table 3). The significantly lowest yield average was recorded in Mezőkövesd (8.94 t/ha), which could be attributed to the fact that, due to the dry weather, the thousand-kernel mass was lowest (327.20 g) and the main ears the shortest (16.86 cm) at this location.

*Table 1*

Average yield and yield component data for maize hybrids in maturity group FAO 200 at each location

| Location | Yield, t/ha | Thousand-kernel mass, g | Kernel/cob ratio, % | No. of kernel rows | Ear length, cm | Protein content, % | Oil content, % | Starch content, % |
|---|---|---|---|---|---|---|---|---|
| Iregszemcse | 9.88 | 319.8 | 86:14 | 15.08 | 18.34 | 7.76 | 3.45 | 66.16 |
| Kaba | 9.87 | 337.0 | 85:15 | 15.35 | 18.93 | 8.46 | 3.60 | 65.34 |
| Martonvásár | 10.52 | 349.1 | 88:12 | 14.93 | 18.22 | 6.98 | 3.45 | 68.01 |
| Mezőkövesd | 7.32 | 324.6 | 86:14 | 14.69 | 17.32 | 8.30 | 3.48 | 66.00 |
| Szarvas | 9.55 | 324.4 | 86:14 | 14.95 | 17.84 | 7.96 | 3.47 | 66.08 |
| $LSD_{5\%}$ | 0.51 | 0.23 | – | 0.21 | 0.31 | 0.07 | 0.01 | 0.22 |
| Mean | 9.43 | 330.98 | 86:14 | 15.00 | 18.13 | 7.89 | 3.49 | 66.32 |

*Table 2*

Average yield and yield component data for maize hybrids in maturity group FAO 300 at each location

| Location | Yield, t/ha | Thousand-kernel mass, g | Kernel/cob ratio, % | No. of kernel rows | Ear length, cm | Protein content, % | Oil content, % | Starch content, % |
|---|---|---|---|---|---|---|---|---|
| Iregszemcse | 10.68 | 338.00 | 88:12 | 14.98 | 18.42 | 7.39 | 3.49 | 67.56 |
| Kaba | 10.08 | 345.10 | 87:13 | 15.17 | 18.67 | 8.40 | 3.62 | 65.57 |
| Martonvásár | 10.84 | 348.00 | 87:13 | 14.87 | 18.76 | 7.35 | 3.45 | 68.25 |
| Mezőkövesd | 8.82 | 329.50 | 88:12 | 14.84 | 16.99 | 7.53 | 3.50 | 66.51 |
| Szarvas | 9.36 | 328.30 | 85:15 | 14.68 | 18.28 | 7.29 | 3.61 | 67.24 |
| $LSD_{5\%}$ | 0.64 | 0.22 | – | 0.22 | 0.26 | 0.07 | 0.02 | 0.30 |
| Mean | 9.96 | 337.78 | 87:13 | 14.91 | 18.22 | 7.59 | 3.53 | 67.03 |

*Table 3*
Average yield and yield component data for maize hybrids in maturity group FAO 400 at each location

| Location | Yield, t/ha | Thousand-kernel mass, g | Kernel/cob ratio, % | No. of kernel rows | Ear length, cm | Protein content, % | Oil content, % | Starch content, % |
|---|---|---|---|---|---|---|---|---|
| Iregszemcse | 9.81 | 331.70 | 87:13 | 15.20 | 18.00 | 7.81 | 3.46 | 66.26 |
| Kaba | 10.02 | 348.00 | 87:13 | 15.49 | 18.54 | 8.37 | 3.50 | 65.55 |
| Martonvásár | 10.23 | 348.70 | 88:12 | 15.31 | 18.95 | 7.09 | 3.43 | 68.15 |
| Mezőkövesd | 8.94 | 327.20 | 88:12 | 14.99 | 16.86 | 7.43 | 3.45 | 66.73 |
| Szarvas | 10.40 | 334.70 | 88:12 | 14.86 | 18.32 | 7.83 | 3.45 | 66.53 |
| $LSD_{5\%}$ | 0.71 | 0.24 | – | 0.23 | 0.25 | 0.06 | 0.01 | 0.11 |
| Mean | 9.88 | 338.06 | 88:12 | 15.17 | 18.13 | 7.71 | 3.46 | 66.64 |

Maize with the greatest thousand-kernel mass (348.70 g) was grown in Martonvásár, where the main ears were also the longest (18.95 cm). The highest protein and oil contents in this maturity group were again found in samples from Kaba (8.37 and 3.50%, respectively), combined with the lowest carbohydrate accumulation (65.55%). By contrast, the lowest protein content (7.09%) and oil content (3.43%) and the highest starch content (68.15%) were measured in the Martonvásár samples.

In the FAO 500 group the best results were obtained in Szarvas (10.92 t/ha) and Martonvásár (10.45 t/ha) (Table 4). The lowest yields were again recorded in Mezőkövesd (8.72 t/ha). In this maturity group the yield and yield components of the hybrids were not as closely correlated with chemical quality traits as in the other three groups. The fact that the highest yield was recorded in Szarvas was due to the fact that the hybrids produced secondary ears, rather than to a greater accumulation of starch, while in Martonvásár the high yield could be attributed to the length of the main ear (20.05 cm), the high thousand-kernel mass (350.3 g) and the total starch content accumulated in the kernels (68.29%). The highest starch content in this group was observed in the Iregszemcse samples, but it was not significantly greater than that of the Martonvásár samples. This starch content (68.48%) was associated with the lowest protein content (7.09%), but the lowest oil content (3.4%) was recorded in Szarvas. The kernels had the highest protein (7.91%) and oil (3.60%) contents under the dry conditions in Mezőkövesd. This was closely correlated with the lowest mean starch content (66.28%).

An analysis was also made for each FAO maturity group of the range over which the chemical quality traits varied between varieties and locations. As can be seen in Tables 5 and 6, the difference in protein content between the varieties was significantly greater in all the maturity groups than between the locations. The difference between the varieties was greatest in the FAO 400 group (2.64%) and smallest in the FAO 300 group (2.11%). The difference between locations for this parameter was greatest in the FAO 200 group (1.48%) and smallest for the FAO 500 hybrids (0.78%).

*Table 4*
Average yield and yield component data for maize hybrids in maturity group FAO 500 at each location

| Location | Yield, t/ha | Thousand -kernel mass, g | Kernel/ cob ratio, % | No. of kernel rows | Ear length, cm | Protein content, % | Oil content, % | Starch content, % |
|---|---|---|---|---|---|---|---|---|
| Iregszemcse | 9.59 | 331.50 | 87:13 | 15.25 | 18.89 | 7.09 | 3.43 | 68.48 |
| Kaba | 9.19 | 359.30 | 86:14 | 15.61 | 19.78 | 7.56 | 3.59 | 67.64 |
| Martonvásár | 10.45 | 350.30 | 86:14 | 15.45 | 20.05 | 7.45 | 3.39 | 68.29 |
| Mezőkövesd | 8.72 | 336.50 | 88:12 | 15.17 | 17.49 | 7.91 | 3.60 | 66.28 |
| Szarvas | 10.92 | 333.10 | 87:13 | 15.17 | 18.71 | 7.87 | 3.40 | 66.81 |
| $LSD_{5\%}$ | 0.66 | 0.24 | – | 0.21 | 0.34 | 0.07 | 0.01 | 0.22 |
| Mean | 9.77 | 342.14 | 87:13 | 15.33 | 18.98 | 7.58 | 3.48 | 67.50 |

*Table 5*
Difference (%) between the effects of varieties and locations on the chemical quality traits of maize hybrids in the FAO 200 and FAO 300 maturity groups

| Traits | FAO 200 | | FAO 300 | |
|---|---|---|---|---|
| | Range | Deviation | Range | Deviation |
| | | Protein content, % | | |
| Between varieties | 9.05–6.60 | 2.45 | 8.57–6.46 | 2.11 |
| $LSD_{5\%}$ | 0.16 | | 0.16 | |
| Between locations | 8.46–6.98 | 1.48 | 8.40–7.29 | 1.11 |
| $LSD_{5\%}$ | 0.07 | | 0.07 | |
| | | Oil content. % | | |
| Between varieties | 3.62–3.42 | 0.20 | 3.62–3.44 | 0.18 |
| $LSD_{5\%}$ | 0.03 | | 0.03 | |
| Between locations | 3.60–3.45 | 0.15 | 3.62–3.45 | 0.17 |
| $LSD_{5\%}$ | 0.01 | | 0.02 | |
| | | Starch content, % | | |
| Between varieties | 67.42–65.37 | 2.05 | 68.33–65.82 | 2.51 |
| $LSD_{5\%}$ | 0.49 | | 0.65 | |
| Between locations | 68.01–65.34 | 2.67 | 68.25–65.57 | 2.68 |
| $LSD_{5\%}$ | 0.22 | | 0.30 | |

In the case of oil content, the difference between varieties was significantly greater in maturity groups FAO 200 and FAO 400, while in the FAO 300 group there was no significant difference between the effects of variety and location. Only for the FAO 500 hybrids was the difference between locations greater than that between the varieties.

The location caused greater differences in the starch accumulation than the variety in the case of FAO 200 hybrids. In the FAO 300 and FAO 500 groups there was no significant difference between the effects, while in the FAO 400 group the difference between the varieties was significantly greater.

*Table 6*
Difference (%) between the effects of varieties and locations on the chemical quality traits of maize hybrids in the FAO 400 and FAO 500 maturity groups

| Traits | FAO 400 | | FAO 500 | |
|---|---|---|---|---|
| | Range | Deviation | Range | Deviation |
| | | Protein content, % | | |
| Between varieties | 9.35–6.71 | 2.64 | 8.66–6.22 | 2.44 |
| $LSD_{5\%}$ | 0.12 | | 0.15 | |
| Between locations | 8.37–7.09 | 1.28 | 7.87–0.09 | 0.78 |
| $LSD_{5\%}$ | 0.06 | | 0.07 | |
| | | Oil content. % | | |
| Between varieties | 3.60–3.36 | 0.24 | 3.54–3.40 | 0.14 |
| $LSD_{5\%}$ | 0.02 | | 0.03 | |
| Between locations | 3.50–3.43 | 0.07 | 3.60–3.39 | 0.21 |
| $LSD_{5\%}$ | 0.01 | | 0.01 | |
| | | Starch content, % | | |
| Between varieties | 67.64–64.47 | 3.17 | 68.60–66.41 | 2.19 |
| $LSD_{5\%}$ | 0.24 | | 0.47 | |
| Between locations | 68.15–65.55 | 2.60 | 68.48–66.28 | 2.20 |
| $LSD_{5\%}$ | 0.11 | | 0.22 | |

When Pearson's correlation coefficients were calculated between the traits it was found that the average yield per hectare was most closely correlated with the thousand-kernel mass (0.72). The yield exhibited a moderate positive correlation (0.42) with the ear length, a negative correlation with the protein and oil contents, and a moderate positive correlation (0.54) with the starch content. A very close positive correlation was observed between the thousand-kernel mass and the starch content (0.91). The protein and oil contents were negatively correlated with all the yield components and exhibited a moderate correlation with each other (0.64). The Pearson coefficient had a value of –0.91 between the protein and starch contents and –0.68 between the oil and starch contents.

Among the chemical quality traits, the starch content was most closely correlated with the yield and all the yield components. As the average yield per hectare increased, there was a corresponding increase in the starch quantity per hectare.

## Discussion

The chemical quality parameters were recorded for almost 100 hybrids, and the correlations of the protein, oil and starch contents with yield and yield components were analysed. It was found that in all the maturity groups the yield was closely correlated with the thousand-kernel mass (0.72). In each maturity group the highest yield averages were associated with the greatest average starch contents (Sprague, 1955; Pásztor et al., 1998), except for the FAO 500 group in the Szarvas location, where the development of secondary ears contributed to the

achievement of the highest yield average. As 80% of the protein content is to be found in the endosperm, where all the carbohydrate is accumulated, it is understandable that maize with the highest starch content had the lowest relative protein content (Fig. 1). A very close correlation was found between the starch content and the thousand-kernel mass (0.91), as previously reported by Bálint (1977). The variety caused greater differences in protein content than the location, as found in previous experiments (Gyenesné-Hegyi et al. 2001). This was also true for the oil content in the FAO 200 and FAO 400 groups, but only in the FAO 400 group in the case of starch content.

More starch was incorporated at wetter locations, where the protein content of the samples was lower, as previously reported by Prokszáné Paplogó et al. (1995) and Gyenesné-Hegyi et al. (2001).

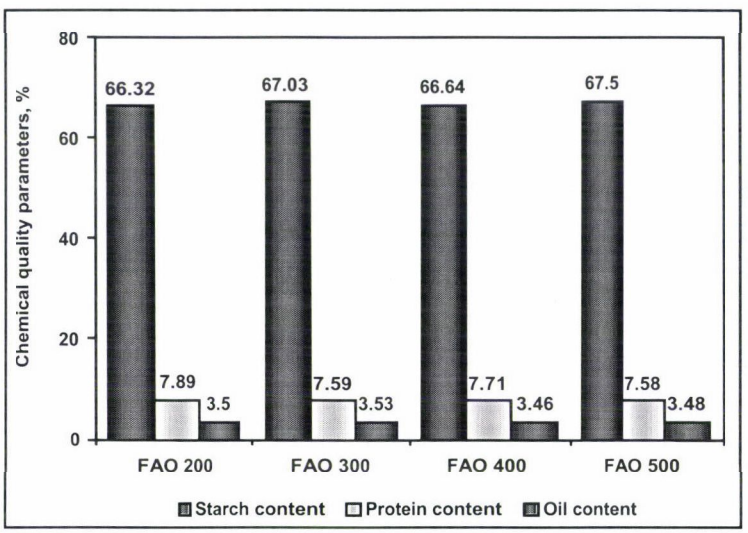



*Fig. 1.* Correlations between chemical quality traits for each maturity group

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# YIELD OF SWITCHGRASS (*Panicum virgatum* L.) AS INFLUENCED BY CUTTING MANAGEMENT

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Switchgrass (*Panicum virgatum* L.) biomass, which is of a good quality in the middle of summer, when cool-season grasses are unproductive, is a very important source of forage. This study measured the influence of the date of first harvest and cutting height on the first and regrowth yields of switchgrass cultivars Blackwell and Cave in Rock. The experiment was conducted in Blacksburg, VA, USA on a Groseclose-Poplimento soil to determine the influence of four dates of harvest and two cutting heights on the yield of switchgrass in 1990, and the influence of the treatments in previous years on the yields in 1991 and 1992. The first yield of both cultivars increased as the date of first harvest was delayed and the cutting height reduced. The regrowth yield of both cultivars declined as the date of first harvest was delayed. A shorter cutting height caused reductions in vigour and yield potential in the second year, whilst in the third year the harvested yield was only 40–50% of that obtained from previously unharvested stands.

**Key words**: switchgrass, *Panicum virgatum*, yield, cutting date, cutting height

## Introduction

Warm season grasses, such as switchgrass (*Panicum virgatum* L.), complement cool season species by filling the summer period of low biomass production, called the summer slump, and therefore help to prevent possible weight loss in cattle. They are also well adapted to marginal soils not suited for other agricultural applications. Although research on the influence of date of first harvest, cutting frequency and height on the yield of warm-season grasses has been conducted since 1930, these grasses are not traditionally grown in Virginia and little is known about their management in more mountainous areas.

Aldous (1930) reported that the cutting of prairie grasses with increasing frequency decreases yield. Harlan and Ahring (1958) suggested that Caddo switchgrass should be cut twice a year and demonstrated that switchgrass

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cultivars give different responses to the same cutting management. Newell (1968) showed that short, blue-green switchgrass lines were less sensitive to cutting frequency than tall, green lines. Berg (1971) concluded that frequent harvesting does not allow switchgrass to store enough energy for winter survival and spring growth. Henry et al. (1976) found that cutting warm season grasses monthly instead of twice in a growing season reduced yield when the cutting height was low, i.e. 80 to 150 mm. Haferkamp and Copeland (1984) concluded that switchgrass plants harvested twice in a season had more aerial, non-rooted shoots than switchgrass plants harvested once. The aerial, non-rooted shoots were more sensitive to winter frost.

Baker et al. (1951) studied the influence of date of first harvest and concluded that early cutting decreased the weight of the stem and allowed increased weed development. Sims et al. (1971) found that early spring harvest did not influence seed head production, while late spring harvest decreased seed head production. Henry et al. (1976) reported that the highest yield of switchgrass could be reached with harvesting dates in July and October or in August and October. Beaty and Powell (1976) concluded that under southern USA conditions switchgrass could be harvested during early spring, because in these areas weed contamination of the switchgrass stand was less severe than in northern areas. Anderson et al. (1989) reported that delaying the date of first cutting weekly increased the yield of the first harvest, but decreased regrowth. The tiller density at first cutting increased as the date of harvest was delayed, but the tiller density at the second cutting was independent of the date of first harvest.

The optimal height of cutting is strongly correlated with the time and frequency of harvest and the maturity and height of the tillers. Researchers emphasise that the removal of the apical meristem weakens switchgrass, reduces yield and increases the number of non-rooted, aerial shoots (Beaty and Powell, 1976; Heidemann and Van Riper, 1967; Sims et al., 1971). Henry et al. (1976) obtained the best stand of switchgrass using a cutting height of 230 mm at all dates of first harvest and the highest yield of switchgrass using a cutting height of 80 mm if the plants were harvested twice in a year. Anderson and Matches (1983) compared the yield and regrowth of switchgrass cut at 80 and 230 mm. They reported that after harvest at 80 mm more than half of the regrowth originated from crown tillers and the rest originated from stem buds, while after harvest at 230 mm primary shoot development continued.

The objective of the present study was to investigate the influence of the date of first harvest and the cutting height on the yield, regrowth, and yield distribution over the growing season, and the influence of the treatments in previous years on subsequent yields of two switchgrass cultivars, Blackwell and Cave in Rock, grown in the mountain areas of Virginia from 1990 to 1992.

## Materials and methods

This field study was conducted on the Research Farm of the Department of Crop and Soil Environmental Sciences in Blacksburg, VA, USA (37°11"N latitude, 80°25"W longitude, 610 m elevation). The Research Farm is located on Groseclose-Poplimento soils, which are deep and well drained, with a clayey subsoil.

Established stands of the switchgrass cultivars Blackwell and Cave in Rock, not harvested prior to 1990, were used for the study. Twenty-eight plots of Blackwell and thirty-two plots of Cave in Rock switchgrass were marked out. Simazine was applied at a rate of 2.2 kg $ha^{-1}$ on 25 May 1990, after early tiller emergence, to control weeds. During the growing season mechanical weed control was not necessary. According to a soil test, the fertility of the soil was maintained at a high level by applying 95 kg $ha^{-1}$ nitrogen fertiliser at the same time as the herbicide.

Seven and eight cutting treatments, respectively, were randomly assigned within four replications of Blackwell and Cave in Rock switchgrass. The influence of four dates of first cut, one harvest interval (four weeks) and two cutting heights on the yield of these cultivars was investigated. Plots with a cutting height of 200 mm were harvested with a rotary mower whilst those with a cutting height of 300 mm were harvested with a sickle bar mower, The harvested plant material was dried to a constant weight in a forced-air oven at 60°C for 36–48 hours, then weighed. A handful of tillers from each treatment were also cut at ground level prior to harvest and the heights of the apical meristems were recorded.

All plots were harvested on 1 November 1990, when all the top growth was dead. In 1991 Simazine and 2,4-D were applied in early May and the plots were again harvested as described above, with the final harvest being carried out on all plots on 21 November. In 1992 a uniform harvest with a cutting height of 200 mm was carried out on 24 June to measure the influence on yield of the treatments in previous years.

The data were analysed by two-way analysis of variance using the general linear model procedure (SAS, 1985) and least significant differences were calculated at the 0.05 probability level.

## Results and discussion

In 1990 the date of first harvest and cutting height affected the first and regrowth yield of both cultivars independently of each other. The first yield of both cultivars increased as the date of first harvest was delayed and the cutting height reduced (Table 1). The difference between the harvested biomass in the 200 and 300 mm cuts increased as the date of first harvest was delayed.

The regrowth yield of Blackwell switchgrass decreased as the date of first harvest was delayed. The dominant apical meristem height was 330 mm on 10 June, 410 mm on 21 June, and 510 mm on 4 July, which suggests that delaying the first cut increased the number of apical meristems removed. However, the apical meristems were always above 300 mm, so the cutting height did not cause any difference in the regrowth yield.

The same influence of the date of first harvest and the cutting height on the regrowth yield was found in the case of Cave in Rock switchgrass. The dominant apical meristem heights were higher for Cave in Rock than for Blackwell. As the first harvest was delayed, the average height of the apical meristems for Cave in Rock increased, being 380 mm on 10 June, 460 mm on 21 June and 560 mm on 4 July. Therefore, the capacity for regrowth and further yield decreased. However, there was no difference between plants cut at 200 or 300 mm, because the dominant apical meristems were always above 300 mm.

*Table 1*

Yield of Blackwell and Cave in Rock switchgrass in 1990, harvested at 200 or 300 mm on different dates for the first cut and after four weeks of regrowth for the second cut. Data are means of four observations; LSD @ $\alpha$=0.05

| Date of harvest | Cultivar, cutting height and yield (g $m^{-2}$) | | | | | |
|---|---|---|---|---|---|---|
| | Blackwell | | | Cave in Rock | | |
| First cut | 200 mm | 300 mm | Mean yield | 200 mm | 300 mm | Mean yield |
| 2 June | 209 | 99 | 154 | 215 | 118 | 160 |
| 11 June | 411 | 291 | 351 | 451 | 356 | 403 |
| 21 June | 646 | 483 | 564 | 698 | 498 | 598 |
| 4 July | – | 683 | – | 1237 | 1039 | 1138 |
| LSD (mean of 4 plots) | 34 | | | | 62 | |
| (mean of 8 plots) | 24 | | | | 44 | |
| Regrowth | | | | | | |
| 28 June | 435 | 400 | 418 | 473 | 388 | 435 |
| 10 July | 194 | 204 | 199 | 172 | 178 | 175 |
| 18 July | 36 | 50 | 43 | 46 | 37 | 42 |
| 1 August | – | 11 | – | 10 | 7 | 9 |
| LSD (mean of 4 plots) | 38 | | | | 47 | |
| (mean of 8 plots) | 27 | | | | 33 | |

Investigations on the influence of the date of first harvest and the cutting height in the first year of harvest on the yields of Blackwell and Cave in Rock switchgrass suggested that the highest regrowth yield can be reached if plants are cut first at the beginning of June, being careful not to remove the apical meristems of the tillers. Across the growing season, plots harvested at a height of 200 mm yielded more harvested material than those cut at a height of 300 mm. However, a cutting height of 200 mm may decrease the vigour of switchgrass, especially if the plants were first harvested at the end of June.

These results are consistent with those of Anderson and Matches (1983), who showed that delaying the date of first harvest reduced the regrowth of switchgrass on the Great Plains. Similarly, Haferkamp and Copeland (1984) observed decreased weight of the primary compound shoot, reduced plant vigour and slow regrowth when switchgrass was mowed in April. A delay in harvest increased the number and weight of secondary, non-rooted and aerial roots and caused less vigour loss than early spring mowing. George and Obermann (1989) concluded from their experiment that higher cutting height made the continuous growth of the apical meristem possible and resulted in greater regrowth. George and Reigh (1987) hypothesised that the best basis for cutting management was the relationship between the height of the leaf tips, upper collar and apical meristem. However, this relationship showed too great a variation between cultivars, harvesting dates, nitrogen levels and years.

In 1991 the yields from the first harvest were very similar irrespective of cutting height, indicating that the 200 mm cutting height in 1990 caused a reduction in vigour and yield potential compared to the 300 mm cutting height (Table 2). From the regrowth harvest following the first cut on 23 May the detrimental influence of the lower cutting height was again evident, as the

highest yields were obtained from the plots cut at 300 mm. However, at later cutting dates this benefit was reversed, with higher regrowth yields being obtained from the later cutting dates. George and Obermann (1989) found the greatest regrowth when the first harvest was taken in early or mid-June and observed that the leaf:stem ratio decreased when the first cut was delayed. It is suggested that in the present trial the plots experiencing greater defoliation as a result of the lower cutting height suffered less evapotranspiration and were better able to regrow during the period of soil water deficit in July, as also reported by Wolf and Parrish (1982).

In 1992 no significant differences between yields from different cutting heights were observed (Table 3).All the treatments produced yields substantially lower than those obtained from areas not harvested in previous years. Although the yields from the previously harvested plots were comparable to those obtained in 1990 and 1991 from a late-June first harvest (approximately 500–600 g $m^{-2}$), the yields from the previously unharvested plots exceeded 1000 g $m^{-2}$.Blackwell suffered a yield reduction of about 40%, whilst in Cave in Rock the yield reduction was approximately 50%.

It was concluded that in Virginia switchgrass can be best utilised for the compensation of cool-season grasses if first cut at the beginning of June at a stubble height which does not result in the removal of apical meristems. A first harvest during the middle or end of June can destroy the structure of the canopy and therefore reduce the yield of regrowth severely. This yield reduction may, however, be mitigated by a lower cutting height, which reduces crop water loss during the period of high soil water deficit in July.

*Table 2*

Yield of Blackwell and Cave in Rock switchgrass in 1991, harvested at 200 or 300 mm on different dates for the first cut and after four weeks of regrowth for the second cut. Data are means of four observations; LSD @ α=0.05

| Date of harvest | Cultivar, cutting height and yield (g $m^{-2}$) | | | | | |
|---|---|---|---|---|---|---|
| | Blackwell | | | Cave in Rock | | |
| First cut | 200 mm | 300 mm | Mean yield | 200 mm | 300 mm | Mean yield |
| 23 May | 204 | 150 | 177 | 193 | 170 | 182 |
| 3 June | 388 | 363 | 374 | 439 | 446 | 442 |
| 17 June | 601 | 625 | 614 | 556 | 623 | 585 |
| 1 July | – | 877 | – | 838 | 903 | 874 |
| LSD (mean of 4 plots) | 47 | | | | 125 | |
| (mean of 8 plots) | 34 | | | | 90 | |
| Regrowth | | | | | | |
| 20 June | 487 | 565 | 525 | 469 | 592 | 531 |
| 1 July | 94 | 101 | 96 | 83 | 76 | 81 |
| 15 July | 90 | 49 | 70 | 87 | 31 | 65 |
| 29 July | – | 90 | – | 141 | 76 | 105 |
| LSD (mean of 4 plots) | 34 | | | | 54 | |
| (mean of 8 plots) | 25 | | | | 38 | |

*Table 3*

Yield of Blackwell and Cave in Rock switchgrass harvested on 24 June 1992 as influenced by different dates of first cut and different cutting heights in 1990 and 1991.Data are means of four observations; LSD @ $\alpha$=0.05

| Date of harvest | Cultivar, cutting height and yield | | | | | |
|---|---|---|---|---|---|---|
| | Actual yield (g $m^{-2}$) | | | Relative yield[1](%) | | |
| Date of first cut in 1990 | 200 mm | 300 mm | Mean yield | 200 mm | 300 mm | Mean yield |
| | | | Blackwell | | | |
| 2 June | 590 | 655 | 623 | 57 | 65 | 61 |
| 11 June | 596 | 650 | 623 | 61 | 65 | 63 |
| 21 June | 601 | 646 | 623 | 58 | 62 | 60 |
| 4 July | – | 619 | 619 | – | 59 | 59 |
| LSD | 174 for means of 4 plots | | | 17 for means of 4 plots | | |
| | | | Cave in rock | | | |
| 2 June | 527 | 572 | 549 | 46 | 49 | 48 |
| 11 June | 601 | 578 | 590 | 53 | 49 | 51 |
| 21 June | 504 | 516 | 509 | 44 | 45 | 44 |
| 4 July | 446 | 563 | 504 | 38 | 49 | 44 |
| LSD | 199 for means of 4 plots | | | 18 for means of 4 plots | | |

[1]Relative yields calculated from treatments with no harvest in previous years. The yields of Blackwell and Cave in Rock were 1080 and 1156 g $m^{-2}$, respectively

The data indicate that any variation in the date of first harvest and the cutting height does not cause any significant difference in the yield and vigour of switchgrass in the years following the first year of harvest. On the other hand, any combination of cutting date and cutting height will lead to a reduced yield in the following year, compared to the yield of the first year.

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# RHEOLOGICAL PROPERTIES OF WHEAT DOUGH IN SOME HEXAPLOID INDIAN WHEAT VARIETIES

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The dough characteristics of fifty popular Indian hexaploid wheat varieties were assessed by testing various rheological properties using a Brabender Farinograph and an Extensograph. These studies were aimed at evaluating the flour quality and functionality of the wheat dough. Based on the rheological dough properties of 50 Indian hexaploid wheat varieties it was recommended that 13 wheats could be useful for blending purposes, 31 varieties could be used for both bread and chapatti making, and the remaining 6 wheats were found suitable for biscuit making. The outcome of the experiments will be useful for plant breeders, millers and bakers.

**Key words**: wheat dough, farinogram, extensogram, viscoelastic properties

## Introduction

Though the utilization of wheat can be categorized into four categories, namely food, feed, pharmacological and industrial, food is the major use of wheat, with close to 75% of world wheat production consumed for this purpose. In recent years, India has produced surplus wheat due to an increase in the area under wheat cultivation and the introduction of high-yielding varieties. However, Indian wheat has not been very suitable for baking and pasta products. To increase the export potential of Indian wheats and to meet international standards it is essential to enhance the quality of Indian wheats. The major characteristics of wheat that relate to its baking quality are its dough properties, which depend on the stiffness or stability, extensibility, mixing tolerance, water absorption and elasticity of the dough (Mariani et al., 1995). Wheat flour should have special qualities, because the functional properties of wheat flour depend not only on various technological processes but also on the quality of the raw material (Anone et al., 2002). Rheological testing helps to evaluate the flour

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quality and the functionality of the wheat dough (Brabender, 1973). Dough properties are severely affected in susceptible varieties attacked by rust diseases, e.g. the dough development time becomes shorter, the mixing tolerance deteriorates and the maximum extensograph resistance becomes lower (O'Brien et al., 1990). These properties ultimately reduce the quality of wheat flour as a whole.

Wheat flour is the major raw material which influences the quality of bakery products. Therefore, the quality control of wheat flour is of paramount importance for the commercial industry. No standard system of classification or grading of wheat and wheat-based products is available. Hence, the alternative method is to analyse, classify and enhance the quality of wheat and wheat-based products from different wheat varieties and to recommend those which possess a high level of desirable quality characters. The flour from milling industries has a naturally lower protein content, alpha amylase activity and sedimentation value, and damaged starch content, making it less suitable for bread making. The baking and pasta industries in India produce bread, biscuits, chapatti and noodles from the same variety. This lack of choice has resulted in the poor growth of the baking and pasta industry. This problem could be overcome by testing the quality of flour and bakery products from different wheat varieties. The present communication reports findings on the various rheological dough properties of different Indian wheat varieties and the classification of these wheats for different baking and pasta products.

## Materials and methods

The fifty hexaploid wheat varieties used in the present study were very popular and extensively grown around the country in different geographical areas, mainly for food. Wheat is unique among all the food grains in having visco-elastic dough properties that are essential in making a range of products, depending on the geographical location. The visco-elastic properties of the wheat dough from these varieties were estimated using a Brabender Farinograph and a Brabender Extensograph according to the standards of the American Association of Cereal Chemists (AACC, 2000). Dough made from the wheat genotypes was analysed for two consecutive years. The Farinograph, which demonstrates dough behaviour when mixed over a period of time, gives an indication of the type of wheat and flour. The consistency of the dough is strongly influenced by its water content and the doughs were compared at the same consistency or mixing torque. The time required for dough development, the mixing tolerance index and the percentage of water absorption are an indication of dough strength, tolerance to work (breakdown) and the amount of water required to produce ideal dough. The farinograms obtained were evaluated for the following parameters: (i) Farinographic water absorption (FWA), (ii) Dough development time (DDT), (iii) Dough stability, (iv) Mixing tolerance index (MTI) / degree of softening.

By contrast the Extensograph records the load-extension curve for a test piece of dough stretched until it breaks. The Extensograph measures the force required to extend dough at a fixed rate of extension. The Extensograms obtained were evaluated for the following parameters: (i) Resistance to extension (R), (ii) Extensibility (E), (iii) Area, (iv) Ratio figure (R/E). Using the Extensograph the flour can be further classified as weak, medium, strong or very strong, based on dough strength. Flours that gave areas of less than 80 $cm^2$ and between 80 and 120 $cm^2$ were classified as weak and medium, while areas of 120–200 $cm^2$ and more than 200 $cm^2$ were classified as strong and very strong dough (Preston and Hoseney, 1991). One-way ANOVA was performed to compare the two-year values of the various rheological properties of the wheat genotypes.

## Results

Dough mixers have been used for over half a century to evaluate wheat quality. In this study Farinograph and Extensograph results were used to characterize the rheology of Indian hexaploid wheat genotypes. The studied wheat genotypes were divided into three quality groups (high, medium and low) based on the rheological properties of the wheat dough. Wheat varieties in the 'high quality' group had strong dough with a high stability time coupled with a lower MTI value. These wheat genotypes also possessed a high quality number, and the Extensograph curve had a greater area, while the R/E values were lower. On the other hand, wheat varieties in the 'low quality' group had weak dough, a low stability time and a higher MTI value. 'Medium quality' wheat varieties showed values intermediate to those of the high and low quality wheat groups. Statistical analysis revealed significant differences between these three groups for various rheological properties. No significant differences were noted between the two years for various rheological properties within each genotype.

The data on the rheological properties (Farinograph and Extensograph) of the dough for 50 Indian hexaploid wheat varieties are presented in Table 1. The Farinograph water absorption values of the 50 wheat varieties in the present study ranged from 53.6 to 67.3%. Higher water absorption (60.2–67.3%) was recorded for 13 wheats; the values were between 55–60% for 31, and in the remaining 6 varieties the water absorption values ranged from 53.6–54.2%. Of the 50 wheat varieties 44 had longer dough development time and stability, while the remaining six varieties had shorter stability time coupled with a low percentage of gluten-forming proteins. Dough development time (DDT) in the Farinograph was long for 44 wheats (range of 4.0–4.4 min), while in six wheat varieties the development period was shorter (range of 3.2–3.5 min). High dough strength and long farinograph stability time (8 min or more) was observed for 13 wheat varieties (range of 8.0–8.3 min). These values were between 5.5 and 7.8 minutes for 31 wheat varieties and 5.0–5.2 minutes for 6 other varieties (Fig. 1A). The Farinograph resistance ranged between 2.3 and 6.3 BU for the 50 wheat varieties, with higher values (3.5 BU and above) for 44 wheats and relatively low values (less than 3.5 BU) for the remaining six varieties. Considerable variation in MTI was observed for the 50 wheat varieties (40–129 BU).

In the present study the dough of six wheat varieties had low MTI value, indicating that the flour of these varieties was soft in nature with low gluten strength. Softness arises when the gluten strength in the wheat flour is low, and the sedimentation is also low in this case. The results are in agreement with those of Autran and Feillet (1987) and Bamidele et al. (1990). The mixing tolerance index was 100 BU or above in six wheat varieties, 85–97 BU in 4 wheats and 58–84 BU in 22 varieties. The flour of 18 wheat varieties had a lower index value (between 40 and 57) for the mixing tolerance of the wheat dough. Out of fifty Indian wheats 18 had a high quality number (81 to 98 on a 0 to 100 scale), while the quality number was medium (63–80) in 26 varieties, and low (50–57) for the remaining six wheat varieties.

*Table 1*
Rheological properties of the dough in fifty Indian hexaploid wheat varieties

| No. | Varieties | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | DW 05 | 60.0 | 4.4 | 8.0 | 6.3 | 45 | 91 | 151 | 430 | 138 | 3.1 |
| 2 | DWR 238 | 55.7 | 4.2 | 7.5 | 4.4 | 59 | 75 | 127 | 705 | 136 | 5.2 |
| 3 | GW 190 | 66.6 | 4.0 | 8.1 | 5.2 | 40 | 85 | 140 | 510 | 85 | 6.0 |
| 4 | GW 313 | 56.9 | 4.2 | 7.4 | 4.9 | 78 | 77 | 130 | 765 | 145 | 5.2 |
| 5 | HD 2735 | 57.3 | 4.1 | 7.5 | 3.3 | 58 | 65 | 118 | 630 | 136 | 4.6 |
| 6 | HD 2737 | 55.4 | 4.2 | 7.3 | 3.7 | 65 | 73 | 124 | 590 | 150 | 3.9 |
| 7 | HD 2741 | 57.8 | 4.3 | 7.0 | 4.4 | 76 | 78 | 91 | 670 | 139 | 4.8 |
| 8 | HD 2744 | 56.6 | 4.4 | 6.9 | 3.8 | 61 | 71 | 129 | 650 | 143 | 4.5 |
| 9 | HD 2754 | 55.0 | 3.5 | 5.2 | 3.1 | 97 | 58 | 86 | 510 | 108 | 4.7 |
| 10 | HI 1476 | 60.7 | 4.1 | 8.0 | 5.8 | 40 | 88 | 141 | 520 | 75 | 6.9 |
| 11 | HI 1479 | 58.5 | 4.0 | 7.8 | 4.7 | 56 | 80 | 128 | 520 | 160 | 3.3 |
| 12 | HP 1731 | 55.7 | 4.3 | 6.8 | 3.1 | 70 | 72 | 110 | 650 | 138 | 4.7 |
| 13 | HP 1835 | 60.3 | 4.4 | 8.0 | 5.3 | 46 | 81 | 147 | 450 | 75 | 6.0 |
| 14 | HS 240 | 67.3 | 4.2 | 8.3 | 6.3 | 49 | 98 | 155 | 430 | 78 | 5.5 |
| 15 | HS 365 | 53.7 | 3.4 | 5.0 | 2.8 | 115 | 56 | 76 | 950 | 177 | 5.4 |
| 16 | HW 2043 | 55.2 | 4.3 | 7.5 | 3.5 | 75 | 74 | 98 | 590 | 139 | 4.2 |
| 17 | HW 2045 | 61.2 | 4.1 | 8.2 | 5.0 | 42 | 89 | 142 | 480 | 80 | 6.0 |
| 18 | HW 3030 | 55.1 | 4.2 | 6.4 | 3.6 | 88 | 59 | 95 | 580 | 120 | 4.8 |
| 19 | HUW 206 | 65.1 | 4.3 | 8.1 | 4.9 | 50 | 86 | 145 | 490 | 89 | 5.5 |
| 20 | HUW 516 | 56.3 | 4.4 | 6.8 | 3.4 | 71 | 80 | 96 | 720 | 145 | 5.0 |
| 21 | HUW 517 | 56.7 | 4.1 | 7.0 | 4.1 | 83 | 63 | 91 | 630 | 140 | 4.5 |
| 22 | HUW 523 | 54.1 | 3.2 | 5.0 | 2.3 | 129 | 52 | 79 | 800 | 180 | 4.4 |
| 23 | HUW 524 | 65.8 | 4.0 | 8.1 | 4.7 | 51 | 82 | 148 | 520 | 76 | 6.8 |
| 24 | HUW 549 | 57.2 | 4.0 | 6.7 | 4.0 | 75 | 67 | 99 | 690 | 135 | 5.1 |
| 25 | K 8027 | 56.4 | 4.0 | 6.3 | 3.6 | 67 | 64 | 97 | 660 | 150 | 4.4 |
| 26 | K 9703 | 55.0 | 4.0 | 5.5 | 4.7 | 91 | 58 | 121 | 680 | 115 | 5.9 |
| 27 | K 9705 | 54.9 | 3.4 | 6.0 | 3.6 | 93 | 60 | 117 | 770 | 119 | 6.5 |
| 28 | K 9707 | 56.6 | 4.4 | 6.6 | 4.9 | 81 | 80 | 93 | 810 | 140 | 5.8 |
| 29 | K 9722 | 64.7 | 4.3 | 8.0 | 4.6 | 47 | 93 | 137 | 480 | 85 | 5.6 |
| 30 | MACS 6092 | 57.3 | 4.1 | 7.5 | 5.1 | 62 | 67 | 130 | 630 | 145 | 4.3 |
| 31 | MP 1039 | 57.1 | 4.4 | 7.6 | 4.8 | 73 | 70 | 120 | 690 | 139 | 5.0 |
| 32 | MP 1095 | 54.0 | 3.2 | 5.2 | 2.9 | 125 | 53 | 83 | 890 | 176 | 5.1 |
| 33 | MP 1103 | 62.5 | 4.0 | 8.0 | 5.2 | 54 | 96 | 150 | 500 | 80 | 6.3 |
| 34 | MP 3034 | 65.3 | 4.4 | 8.2 | 4.6 | 49 | 87 | 147 | 510 | 85 | 6.0 |
| 35 | NIAW 391 | 60.0 | 4.3 | 7.8 | 5.7 | 55 | 81 | 138 | 780 | 158 | 4.9 |
| 36 | NW 1073 | 61.9 | 4.2 | 7.7 | 5.5 | 57 | 82 | 135 | 760 | 155 | 4.9 |
| 37 | PBW 460 | 55.2 | 4.1 | 7.6 | 3.9 | 67 | 71 | 120 | 610 | 150 | 4.1 |
| 38 | PBW 462 | 54.1 | 3.4 | 5.1 | 2.7 | 117 | 57 | 81 | 710 | 179 | 4.0 |
| 39 | Raj 3984 | 56.5 | 4.2 | 6.5 | 3.8 | 74 | 67 | 119 | 530 | 135 | 3.9 |
| 40 | Raj 3985 | 64.6 | 4.4 | 8.0 | 4.8 | 43 | 91 | 146 | 495 | 85 | 5.8 |
| 41 | Raj 3989 | 56.1 | 4.3 | 7.3 | 4.1 | 70 | 75 | 133 | 700 | 150 | 4.7 |
| 42 | Raj 3992 | 55.3 | 4.0 | 7.1 | 4.4 | 64 | 72 | 124 | 630 | 140 | 4.5 |
| 43 | RSP 305 | 61.8 | 4.4 | 8.3 | 4.6 | 52 | 88 | 143 | 470 | 89 | 5.3 |
| 44 | UP 2477 | 59.0 | 4.1 | 7.7 | 4.5 | 57 | 63 | 136 | 720 | 157 | 4.6 |
| 45 | UP 2482 | 55.7 | 4.3 | 6.8 | 4.1 | 84 | 74 | 116 | 690 | 139 | 5.0 |
| 46 | VL 738 | 53.6 | 3.3 | 5.0 | 2.5 | 108 | 55 | 78 | 810 | 180 | 4.5 |
| 47 | VL 852 | 54.2 | 3.2 | 5.1 | 2.3 | 100 | 51 | 77 | 720 | 177 | 4.1 |
| 48 | WH 705 | 57.7 | 4.0 | 6.9 | 4.7 | 66 | 78 | 113 | 710 | 142 | 5.0 |
| 49 | WH 707 | 56.5 | 4.3 | 7.4 | 4.6 | 75 | 60 | 94 | 590 | 148 | 4.0 |
| 50 | WH 714 | 59.2 | 4.4 | 7.7 | 4.2 | 56 | 83 | 110 | 630 | 159 | 4.0 |
| | SED | 2.45 | 0.69 | 0.67 | | 2.72 | 3.22 | 14.59 | 66.91 | 0.80 | 0.68 |
| | CD | 4.89 | 1.38 | 1.32 | 5.24 | 5.40 | 6.39 | 28.95 | 132.8 | 1.60 | 1.34 |
| | (P = 0.05%) | ** | ** | ** | ** | ** | ** | ** | ** | ** | NS |

1: Water absorption (%); 2: Dough development time (min); 3: Stability time (min); 4: Farinograph resistance; 5: Mixing tolerance index; 6: Quality number; 7: Area ($cm^2$); 8: Extensograph resistance; 9: EXTN (mm); 10: R/E; NS= non significant

The doughs tested in the present study showed decreased resistance to extension in six wheats, so these varieties were only suitable for biscuit-making. The remaining 44 varieties had high to medium resistance to extension, with greater smoothness, and were hence suitable for all purposes, including bread-making, chapatti-making, and blending with poor quality flour. The resistance to extension recorded with the Extensograph ranged between 470 and 950 BU for the dough of the 50 wheat varieties. Higher resistance to extension was observed for six wheats, ranging from 580–950 BU, while for the remaining wheats the resistance to extension was relatively lower (470–520 BU). Among the 50 wheat varieties, the extensibility of the dough ranged from 75–185 BU, with values higher than 150 BU for 6 wheat varieties, 90–150 BU for 31 wheats, and 75–89 BU for the remaining 13 wheat varieties (Fig. 1B). The ratio between resistance to extensibility and dough extension was lower than 5.0 in 37 wheat varieties and above 5.0 for the remaining 13 wheat varieties (Fig. 1C). A greater area under the Extensograph curve indicated increased dough strength. The area under the Extensograph curve was above 140 $cm^2$ for 13 wheat varieties, 89–136 $cm^2$ for 27 varieties and 76–88 $cm^2$ for the remaining 10 wheat varieties.

## Discussion

The higher the water absorption, the stronger the flour and vice versa. The farinographic water absorption is the amount of water required to develop dough of the desired consistency, which in turn causes the curve to rise to the 500 BU consistency line at the peak of mixing, i.e. when the gluten is fully developed. The dough development time is the time from the first addition of water to the development of maximum dough consistency with minimum mobility. A long dough development time indicates strong flour containing a high percentage of gluten-forming proteins. Dough stability time is the major index for dough strength. Flour with longer stability is best suited for bread-making, while flour with a short stability time can be used for making biscuits and chapatti. A long dough stability time gives great strength to the gluten and makes it resistant to mechanical damage. The mixing tolerance index (MTI) is another parameter used for indicating flour-mixing requirements. Strong flour has a lower MTI value and weak flour a higher value (Kaur et al., 1998). Extensibility is the total length of the Extensograph curve obtained with an extensometer. This parameter is used to estimate the resistance of the dough to extension. A longer curve is an indicator of low resistance to extension, which means that the dough is weak and has less elastic properties, making it useful only for biscuit-making. On the other hand, a shorter curve indicates high resistance to extension, giving strong dough with more elastic properties, suitable for bread-making. Bread-making dough shows strong resistance to stretching because of its elastic glutenin. After limited extension the dough breaks. By contrast, biscuit dough is much less resistant to stretching and also extends further before breaking (Chen and Rasper, 1982).

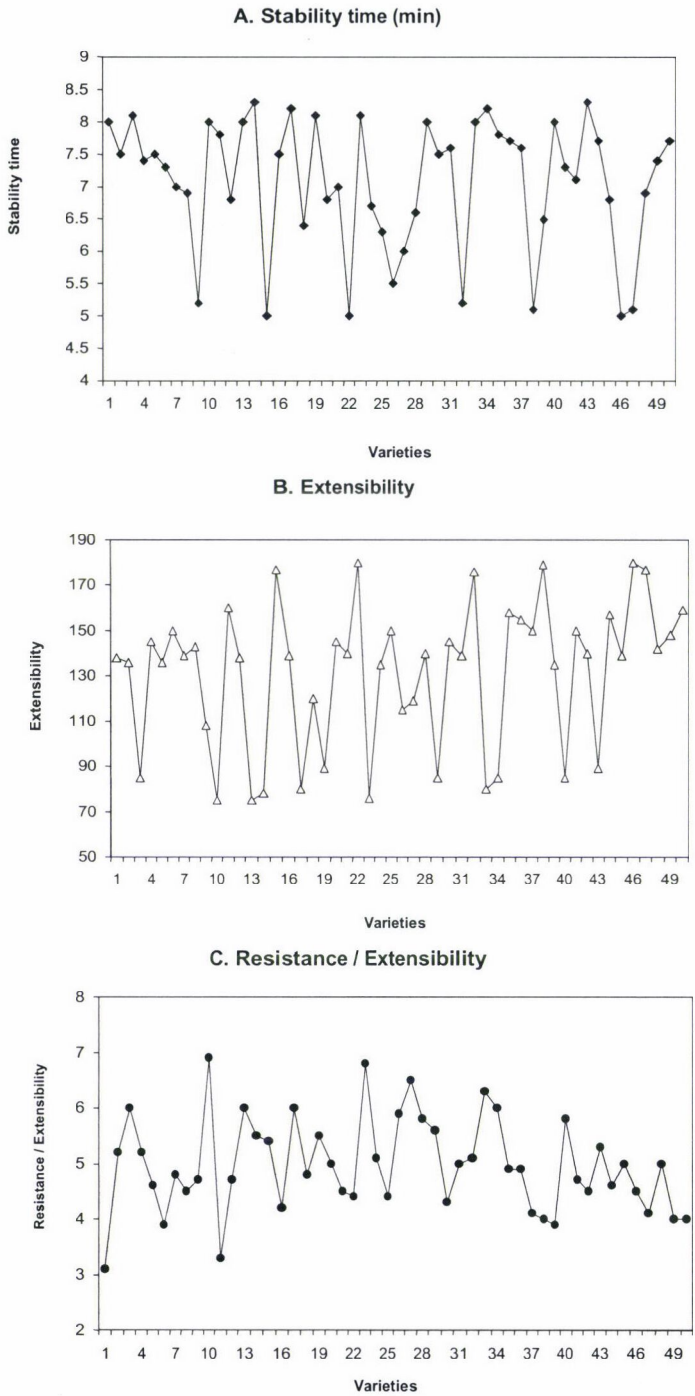



*Fig. 1.* Rheological properties of the dough in Indian hexaploid wheat varieties

It was suggested that bread-making wheat was quite unsuitable for biscuit-making and vice versa. The farinographic and extensographic parameters of the dough of fifty Indian hexaploid wheat varieties revealed that 26% of the Indian wheats are suitable for blending purposes, while the majority of them (62%) can be used for both bread and chapatti making. Only a few varieties (12%) were found to be suitable for biscuit-making. Studies of this nature will be helpful for the milling industry, to facilitate the blending of high quality wheat flour with low quality flour, for the baking industry to assess the suitability of various wheat genotypes for different types of baking and pasta products, and also for plant breeders for screening genotypes with the desired quality.

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# IMPORTANCE OF PLANT GROWTH-PROMOTING RHIZOBACTERIA IN ENHANCING THE SEED GERMINATION AND GROWTH OF WATERMELON ATTACKED BY FUNGAL PATHOGENS

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In the present study, seven isolates of plant growth-promoting rhizobacteria were used for seed treatment to suppress seedling diseases caused by fungi. Their effect on the seed germination and seedling vigour of watermelon was also studied. Among them INR-7 was able to inhibit a broad range of fungal species, GBO3 and IPC-11 were found to be effective against *Fusarium* spp. and *Didymella bryoniae*, while isolates SE-34 and T-4 were effective against *Myrothecium* species and also reduced the development of symptoms on the seedlings. Isolates GBO3, IPC-11 and INR-7 increased seed germination and seedling vigour to the greatest extent.

**Key words:** watermelon, fungal disease, PGPR, fungal inhibition, promotion of seed germination and growth

## Introduction

Watermelon is an important crop, commonly grown for its fruits especially during summer. Seed-borne fungal pathogens play a major role in seedling diseases and cause considerable losses in the yield of the crop in the field. Many chemical fungicides are used to control these diseases. The control of plant diseases using antagonistic bacteria is now considered as a promising alternative method that could reduce the use of hazardous chemical fungicides or bactericides. Bio-pesticides are up to 50% cheaper than synthetic pesticides. They are ecofriendly in nature, have a high cost-benefit ratio and do not pose the risk of the pathogen developing resistance. They are easy to apply and are compatible with bio-fertilizers. Many root-colonizing bacteria are known to promote plant growth by producing gibberellins, cytokinin and indole acetic acid (Dubeikovsky et al., 1993) and are hence called Plant Growth-Promoting Rhizobacteria (PGPR) (Kloepper and Schroth, 1978). Several PGPR strains have

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been reported to control various fungal (Van Peer et al., 1991), bacterial (Liu et al., 1995) and viral diseases (Maurhofer et al., 1994; Raupach et al., 1996). The mechanisms of biological control by PGPR strains generally involve the production of siderophores (Kloepper et al., 1980; Becker and Cook, 1988), hydrogen cyanide (Ahl et al., 1986) and lytic enzymes (Sneh et al., 1984; Jones et al., 1986), the biosynthesis of antibiotics (Howie and Suslow, 1991) and competition for substrates (Elad and Chet, 1987). PGPR are also known to act as inducers of systemic resistance in plants (Alstrom, 1991; Van Peer et al., 1991; Wei et al., 1991; Zhou and Paulitz, 1994; Raupach et al., 1996). Since several studies have indicated the stimulatory properties of PGPR, various PGPR isolates were used for seed treatment against fungal infection in the present investigation, with a view to enhancing plant growth and yield.

## Materials and methods

Seed samples of a popular variety of watermelon, namely Sugar Baby, were obtained from the seed company in Bangalore and stored in a polyethylene bag at 28°C until further use. The seeds were surface sterilized with 0.1% mercuric chloride solution for 3 min, followed by three washes in sterilized distilled water. The surface-sterilized seeds were then air-dried and the seeds were subjected to the standard blotter method, the paper towel method and pot experiments to study the incidence of mycoflora, germination, seedling vigour and disease symptoms.

Seven different strains of plant growth-promoting rhizobacteria (PGPR), namely *Bacillus pumilus* (INR-7), *B. subtilis* (GBO-3), *B. subtilis* (IN937b), *B. pumilus* (SE-34), *Brevibacillus brevis* (IPC-11), *B. pumilus* (T-4) and *B. amyloliquefaciens* (IN937a), were obtained from authentic stock cultures maintained in the Department of Applied Botany and Biotechnology, Mysore and were grown separately in nutrient yeast broth (NYB) medium maintained at 30°C for 48 h. After 48 h of incubation, the cultures were centrifuged at 10,000 rpm for 10 minutes, the pellets obtained were suspended in distilled water and the inoculum load was adjusted to $1 \times 10^8$ CFU/ml based on spectrophotometry (OD 0.45 @ 610 nm). The seeds were separately soaked overnight in the culture suspension and air-dried. Control seeds were soaked in distilled water.

In the other set, treated seeds were then plated equidistantly on three layers of wet blotters on Perspex plates and incubated at 22 ± 2°C under alternate cycles of 12/12 hours near-ultraviolet light and darkness for 7 days. On the 8th day of incubation the seeds were evaluated for mycoflora following the procedures of ISTA (Anonymous, 1996). The dominant fungi, such as *Alternaria cucumerina* (Ellis & Everhart) Elliot, *Myrothecium verrucaria* (Albertini & Schewein) Detmar ex Fr., *Myrothecium roridum* Tode ex Fr., *Didymella bryoniae* (Awersw.) Rehm, *Fusarium oxysporum* Schlecht. Emend. Snyder & Hansen, *Fusarium solani* (Mart.) Apple & Wollenw. emend. Snyder & Hansen, *Fusarium equiseti* (Carda) Sacc. and *Fusarium verticilloides* (Sheldon), were recorded.

Treated seeds were also subjected to the paper towel method, in which the seeds were plated equidistantly between wet blotters and incubated at 22 ± 2°C under 12/12 hours alternate cycles of light and darkness for 14 days. After incubation, the seed germination percentage and root-shoot lengths of the seedlings were assessed and the vigour index was calculated based on the formula prescribed by Abdul Baki and Anderson (1973).

In the third set both treated and untreated seeds were sown separately in sandy soil in 30 cm earthen pots (10 seeds/pot) and maintained under greenhouse conditions. On the 15$^{th}$ day seedlings showing symptoms such as cotyledonary necrosis, damping-off or wilt were recorded and their incidence was tabulated in comparison with the control. The resultant data from repeated experiments were combined and analysed statistically based on ANOVA followed by Duncan's Multiple Range Test.

## Results and discussion

Table 1 revealed the varied occurrence of fungi in the seeds of watermelon variety Sugar Baby due to PGPR treatment. Among the PGPR isolates used for seed treatment, INR isolates showed the broadest range of inhibition of the pathogens on the seeds. GBO3 was also found to be effective against *Fusarium oxysporum*. The IN937b and T-4 isolates were found to be least effective against this pathogen. The GBO3 and IPC-11 isolates resulted in the inhibition of *Didymella bryoniae,* while *Alternaria cucumerina* and *Acremonium cucurbitacearum* were effectively reduced by IPC-11 and IN937b, whereas the incidence of *Myrothecium roridum* and *M. verrucaria* was found to be inhibited by isolates T-4 and SE-34. Table 2 illustrates the phytostimulatory properties of the PGPR strains, all of which improved seed germination and seedling vigour, the best results being recorded for isolates GBO3 and INR-7, which enhanced seedling germination by 15% over the control.

All the isolates of PGPR reduced the disease symptoms. GBO3, IPC-11 and INR-7 effectively reduced cotyledonary necrosis, which is caused by *Didymella bryoniae* (Table 3). T-4 and SE-34 were very effective in reducing damping-off, caused by *Myrothecium* species. Wilt caused by *Fusarium* species was also effectively reduced by GBO3 and INR-7.

The data presented in Tables 1 and 3 are closely related to each other. The massive accumulation of phytoalexins and phenolic compounds, the increased accumulation of PR proteins and peroxidase, increased levels of mRNAs encoding phenylalanine ammonia-lyase (PAL), chalcone synthase and PR1a protein, and enhanced lignification have been reported in plants following treatment with PGPR strains (Hynes and Lazarovits, 1989; Van Peer et al., 1991; Zdor and Anderson, 1992; M'Piga et al., 1997).

*Table 1*
Occurrence of fungi in the PGPR-treated seeds of watermelon

| PGPR isolates | Percentage incidence of seed mycoflora | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *A. c.* ±SE | *Al. c.* ±SE | *D. b.*±SE | *F. o.* ±SE | *F. s.* ±SE | *F. e.* ±SE | *F. v.* ±SE | *M. r.* ±SE | *M. v.* ±SE |
| GBO3 | 10± 0.4$^{c}$ | 13±0.4$^{c}$ | 18±0.4$^{e}$ | 18±0.6$^{f}$ | 24±0.8$^{e}$ | 12±0.6$^{e}$ | 3±0.2$^{f}$ | 14±0.7$^{cd}$ | 16±0.7$^{de}$ |
| INR-7 | 8±0.3$^{d}$ | 11±0.5$^{bc}$ | 16±0.5$^{f}$ | 20±0.7$^{e}$ | 23±0.8$^{f}$ | 10±0.5$^{f}$ | 5±0.3$^{e}$ | 11±0.6$^{d}$ | 17±0.8$^{e}$ |
| IPC-11 | 5±0.3$^{e}$ | 9±0.3$^{cd}$ | 18±0.6$^{e}$ | 29±0.8$^{d}$ | 23±0.9$^{f}$ | 17±0.8$^{d}$ | 5±0.3$^{e}$ | 16±0.8$^{b}$ | 22±0.8$^{c}$ |
| IN937a | 13±0.8$^{b}$ | 14±0.5$^{c}$ | 27±1.0$^{b}$ | 25±0.6$^{de}$ | 27±1.1$^{d}$ | 21±0.9$^{b}$ | 11±0.6$^{b}$ | 13±0.7$^{c}$ | 20±0.8$^{d}$ |
| IN937b | 7±0.5$^{d}$ | 8±0.7$^{d}$ | 25±0.9$^{c}$ | 36±1.1$^{b}$ | 33±1.2$^{c}$ | 15±0.7$^{de}$ | 9±0.5$^{cd}$ | 10±0.5$^{de}$ | 24±0.9$^{b}$ |
| T-4 | 10±0.5$^{c}$ | 16±0.8$^{ab}$ | 21±0.8$^{d}$ | 31±1.0$^{c}$ | 28±1.0$^{de}$ | 19±0.9$^{c}$ | 10±0.6$^{c}$ | 7±0.3$^{f}$ | 11±0.5$^{f}$ |
| SE-34 | 12±0.7$^{bc}$ | 15±0.8$^{b}$ | 28±0.9$^{b}$ | 27±0.9$^{d}$ | 37±1.4$^{b}$ | 17±0.8$^{d}$ | 7±0.4$^{d}$ | 9±0.5$^{e}$ | 13±0.6$^{g}$ |
| Control | 18±0.6$^{a}$ | 21±0.9$^{a}$ | 37±1.1$^{a}$ | 42±1.5$^{a}$ | 46±1.8$^{a}$ | 25±1.0$^{a}$ | 15±0.7$^{a}$ | 23±0.9$^{a}$ | 28±1.0$^{a}$ |

*A. c.: Acremonium cucurbitacearum; Al. c.: Alternaria cucumerina; D. b.: Didymella bryoniae; F. o.: Fusarium oxysporum; F. s.: Fusarium solani; F. e.: Fusarium equiseti; F. v.: Fusarium verticilloides; M. r.: Myrothecium roridum; M. v.: Myrothecium verrucaria*; GBO3= *Bacillus subtilis*, INR-7 = *B. pumilus*, IPC-11 = *Brevibacillus brevis*, IN937a = *B. amyloliquefaciens*, IN937b = *B. subtilis*, T-4 = *B. pumilus* and SE-34 = *B. pumilus*; Data based on 400 seeds of four replicates of 100 seeds each. According to Duncan's Multiple Range Test (DMRT), values followed by different superscripts are significantly different at $P \leq 0.05$. SE = Standard error of the mean

*Table 2*
Influence of PGPR isolates on seed germination and seedling vigour of watermelon

| PGPR isolates | Seed germination* (%) ±SE | MRL±SE | MSL±SE | VI±SE |
|---|---|---|---|---|
| GBO3 | 95±0.6$^{a}$ | 12.8±0.1$^{a}$ | 15.7±0.9$^{a}$ | 2706±44$^{a}$ |
| INR-7 | 93±0.4$^{a}$ | 12.5±0.09$^{b}$ | 15.8±0.9$^{a}$ | 2632±42$^{b}$ |
| IPC-11 | 88±0.2$^{bc}$ | 12.3±0.1$^{abc}$ | 15.5±1.0$^{c}$ | 2446±41$^{d}$ |
| IN937a | 85±0.5$^{c}$ | 12.1±0.07$^{c}$ | 15.3±1.1$^{d}$ | 2329±22$^{cd}$ |
| IN937b | 90±0.9$^{b}$ | 12.4±0.08$^{ab}$ | 15.6±1.0$^{ab}$ | 2520±37$^{c}$ |
| T-4 | 80±0.6$^{e}$ | 11.5±0.05$^{de}$ | 14.7±0.09$^{e}$ | 2096±29$^{de}$ |
| SE-34 | 83±0.4$^{d}$ | 11.8±0.05$^{cd}$ | 15.0±0.05$^{de}$ | 2224±24$^{d}$ |
| Control | 76±0.3$^{f}$ | 11.3±0.08$^{e}$ | 14.5±0.08$^{d}$ | 1961±46$^{e}$ |

*Data based on the paper towel method; Data were recorded 14 days after sowing based on the average of 400 seeds of four replicates for each treatment. According to Duncan's Multiple Range Test (DMRT), values followed by different superscripts were significantly different at $P \leq 0.05$. MRL=Mean root length, MSL=Mean shoot length, VI=Vigour index and SE=Standard error of the mean.

*Table 3*
Role of PGPR isolates in the reduction of disease symptoms in watermelon under greenhouse conditions

| PGPR isolates | Percentage incidence of disease symptoms | | |
|---|---|---|---|
| | Cotyledonary necrosis ±SE | Damping off ±SE | Seedling wilt ±SE |
| GBO3 | 4±0.3$^{f}$ | 16±0.9$^{d}$ | 6±0.3$^{h}$ |
| INR-7 | 7±0.4$^{e}$ | 13±0.9$^{e}$ | 9±0.5$^{g}$ |
| IPC-11 | 5±0.5$^{de}$ | 25±1.2$^{b}$ | 13±0.8$^{f}$ |
| IN937a | 9±0.7$^{dc}$ | 16±0.8$^{d}$ | 19±0.9$^{d}$ |
| IN937b | 14±1.0$^{c}$ | 20±1.0$^{c}$ | 17±1.0$^{e}$ |
| T-4 | 10±0.9$^{d}$ | 9±0.5$^{ef}$ | 26±1.2$^{b}$ |
| SE-34 | 12±0.8$^{b}$ | 8±0.5$^{f}$ | 23±1.3$^{c}$ |
| Control | 27±1.2$^{a}$ | 35±1.6$^{a}$ | 48±2.1$^{a}$ |

Data were recorded on the 14$^{th}$ day after sowing based on the average of 200 seeds of four replicates for each treatment. According to Duncan's Multiple Range Test (DMRT), values followed by different superscripts were significantly different at $P \leq 0.05$. SE= Standard error of the mean

Plant growth-promoting rhizobacteria (PGPR) are free-living bacteria having a beneficial effect on plants as they enhance emergence, colonize roots and stimulate growth (Kloepper et al., 1988). In recent years, the concept of using PGPR for the promotion of plant growth is gaining worldwide acceptance (Kloepper et al., 1991). PGPRs stimulate host plant growth through several possible mechanisms, including biological control (Pleban et al., 1995), induced systemic resistance to plant pathogens (Benhamou, 1996; Hallmann et al., 1997), phytohormone production, and the improvement of nutrient and water uptake (Pleban et al., 1995; Nowak and Lazarovits, 1997). Some of these organisms can be utilized in agricultural and horticultural practice for the purpose of transplantation, protection against diseases, improvement, establishment and

overall performance (Wei et al., 1996; Nowak et al., 1998; Bharath et al., 2005). They may also improve plant performance in stress environments and consequently enhance yields (Nowak et al., 1998; Frommel et al., 1991). The present findings are in agreement with these results. Similar observations were made by Raupach and Kloepper (1998), who used GBO3 and INR7 isolates for seed treatment in the management of cucumber pathogens such as *Colletotrichum orbiculare*, *Pseudomonas syringae* pv. *lachrymans* and *Erwinia tracheiphila* under greenhouse conditions. The present results suggest that PGPR will also have promising effects in the management of seed-borne diseases of watermelon.

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# EFFECT OF DIFFERENT SOWING DENSITIES ON SOME CHARACTERISTICS OF *Isatis tinctoria* L. AND *Isatis constricta* DAVIS AND ON THE RECOVERY OF INDICAN

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The study reports the effects of four sowing densities (40 × 10, 40 × 20, 60 × 10 and 60 × 20 cm) on the agronomic characteristics of *Isatis tinctoria* and *I. constricta* under the rainfed conditions of South Eastern Anatolia. Wide row spacings of 60 × 10 or 60 × 20 cm were effective in obtaining maximum number of leaves per plant, leaf length, leaf width, petiole length, stem diameter, fruit length, 1000 fruit weight and 1000-seed weight. However, narrow row spacing (40 × 10 or 40 × 20 cm) led to maximum values of fresh and dry leaf yield 10 $m^{-2}$, plant height, fruit yield and fruit length, minimum hull content, and the highest indican percentage and indican yield $m^{-2}$. This information will be helpful for the economical cultivation of these plants under the rainfed conditions of South Eastern Anatolia.

**Key words:** woad, sowing density, leaf yield, seed yield, indican

## Introduction

Dyes have always been a source of fascination and importance in the life of people. With the discovery of synthetic dyes at the end of the 19th century, natural dyes gradually disappeared and traditional dyeing methods fell into oblivion (Anonymous, 1997). The genus *Isatis* has over 50 species that grow in a range of climatic and soil conditions. Several species are cultivated and are used as dyes or for medicinal purposes. *Isatis tinctoria* L. (woad, family *Brassicaceae*) and *I. constricta* L. are ancient dyes and medicinal herbs and are economically the most important plants in the carpet industry of Turkey. *Isatis* spp. are native to the steppe and desert zones of the Caucasus, Central Asia to eastern Siberia and Western Asia, and are widely distributed in south-west Asia, Uzbekistan, Russia, Tajikistan, Mongolia, Pakistan, Korea, Kazakhstan, Japan (Shu, 2001) and Europe (Tan, 2002), and have been naturalised elsewhere (e.g. North America) (Moazzeni and Zarre, 2006). *Isatis* spp. were widely cultivated

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in Europe from the 12th to the 17th century as a source of indigo (Brunello, 1973; Clark et al., 1993; Hurry, 1930).

Woad produces isatan B in its leaves, which, when exposed to air, forms a blue compound called indigo. The indigo precursor, indoxyl, is mainly in the form of the glucoside indican (indoxyl-b-D-glucoside), or the ester isatan B (indoxyl-5-ketogluconate) (Anonymous, 1997).

The use of woad persisted as a trusted remedy both for diseases and for surgical disorders from ancient times to the present (Hurry, 1930). The Chinese and East Indians also used woad leaves and roots as a broad-spectrum antibiotic and as a treatment for many different infections and inflammations (Hurry, 1930).

The provision of low cost plant material of high quality for agriculture is an important factor for the revival of natural dyes. For this purpose it is necessary to optimize cultivation techniques for the recovery of important dyes.

To our knowledge, no investigations on the agronomical properties and dyestuff content (indican) of *I. tinctoria* and *I. constricta* have been reported to date. The present study aimed to determine the effect of different sowing densities on agronomic characters such as fresh leaf yield, dry leaf yield, fruit yield and recovery of dyestuff (indican), and to identify suitable crop improvement techniques for the commercial recovery of dyestuff (indican) from these plants.

## Materials and methods

*Materials*

*I. tinctoria* L. seeds were obtained from the Department of Field Crops, Faculty of Agriculture, University of Ankara, Turkey, while *I. constricta* Davis seeds were collected from the Maden district between the Diyarbakir and Elazıg provinces of Turkey. The experiment was carried out in the 1999–2000 and 2000–2001 crop seasons in the experimental area of the Department of Field Crops, Faculty of Agriculture, University of Dicle, Diyarbakir, Turkey.

Meteorological data for 1999 and 2000 showed that the lowest monthly mean temperature was recorded in January and the highest mean temperature in July in both years. The experimental location had a long-term mean temperature of 15.8°C. The mean temperature of the first experimental year (16.5°C) was higher than that of the second experimental year (15.9°C) and the long term mean. The long-term mean relative humidity of 53.8% was higher than that of the first (48.4%) and second (46.5%) experimental years. The long-term mean precipitation (40.7 mm) was also higher than in the experimental years (21.7 mm and 31.9 mm, respectively). The long-term mean, 1999 and 2000 values of temperature, relative humidity and precipitation during the January–June period were 12.1, 13.3 and 12.6°C, 60.9, 56.8 and 53.0%, and 54.7, 35.5 and 33.2 mm, respectively (Table 1).

*Methods*

Seeds of *I. tinctoria* and *I. constricta* were sown directly into well-prepared field beds on 16 February 1999 in triple row plots 6 m in length with inter- and intrarow spacing of 40 × 10, 40 × 20, 60 × 10 and 60 × 20 cm. Plot size was 9.6 $m^2$ (2.4 × 4 m) for 40 cm row spacing and 14.4 $m^2$ (3.6 m × 4 m) for 60 cm. Sowing was carried out at a depth of 1–2 cm by hand dropping 2–3 seeds (hard-shell or fruit) per hole. During the vegetation period, the plots were weeded and irrigated as required. Before sowing, manure was added to the soil at the rate of 20 t $ha^{-1}$ and homogenised.

*Table 1*

Meteorological observations on the experimental area in 1999 and 2000

| Months | Long-term means | | | 1999 | | | 2000 | | |
|---|---|---|---|---|---|---|---|---|---|
| | Temp. (°C) | R. hum. (%) | Precip. (mm) | Temp. (°C) | R. hum. (%) | Precip. (mm) | Temp. (°C) | R. hum. (%) | Precip. (mm) |
| January | 1.7 | 76.5 | 73.6 | 4.5 | 71 | 15.6 | 1.3 | 74 | 70.9 |
| February | 3.5 | 72.5 | 67.0 | 5.3 | 67 | 45.5 | 2.5 | 65 | 58.2 |
| March | 8.2 | 66.0 | 67.9 | 8.1 | 65 | 52.0 | 7.0 | 64 | 30.7 |
| April | 13.8 | 63.3 | 70.5 | 13.6 | 64 | 76.1 | 15.3 | 57 | 33.0 |
| May | 19.2 | 56.2 | 42.1 | 21.0 | 43 | 22.4 | 21.3 | 37 | 6.1 |
| June | 26.0 | 31.2 | 6.9 | 27.3 | 31 | 1.1 | 28.0 | 21 | 0.3 |
| Mean (Jan.–Jun.) | 12.1 | 60.9 | 54.7 | 13.3 | 56.8 | 35.5 | 12.6 | 53.0 | 33.2 |
| July | 31.0 | 27.3 | 0.6 | 31.4 | 26 | 0.9 | 33.4 | 13 | 0.0 |
| August | 30.3 | 27.5 | 0.4 | 30.6 | 27 | 0.0 | 30.4 | 20 | 0.0 |
| September | 24.8 | 31.7 | 2.7 | 24.4 | 37 | 10.5 | 24.7 | 27 | 0.9 |
| October | 17.1 | 48.3 | 31.1 | 17.6 | 43 | 2.7 | 16.7 | 47 | 35.1 |
| November | 9.6 | 67.5 | 54.1 | 9.8 | 41 | 1.9 | 9.4 | 54 | 34.0 |
| December | 4.1 | 77.0 | 71.5 | 4.8 | 66 | 31.5 | 4.3 | 79 | 113.6 |
| Mean (Jul.–Dec.) | 19.4 | 46.6 | 26.7 | 19.7 | 40.0 | 7.9 | 19.3 | 40.0 | 30.6 |
| Mean (12 months) | 15.8 | 53.8 | 40.7 | 16.5 | 48.4 | 21.7 | 15.9 | 46.5 | 31.9 |

Temp. = Temperature, R. hum. = Relative humidity, Precip. = Precipitation; Source: State Meteorology Institute, Diyarbakir, Turkey (2000)

The leaves were harvested on 1–4 July 1999 at the optimum stage of leaf development. The plants were harvested at the soil level on 30–31 May 2000 using scissors and dried in a cool shady place before threshing to obtain the fruits.

Both species were evaluated during the first year for the number of leaves per plant, leaf length, leaf width, petiole length, fresh leaf yield and dry leaf yield. Other agronomic characteristics, such as plant height, stem diameter, first branch height, fruit yield, fruit length, fruit width, thousand fruit weight, thousand seed weight and hull content of the fruit were measured at harvest during the second year.

The general linear model was used to perform univariate analysis of variance to determine the effect of sowing density on various agronomic traits of *I. tinctoria* and *I. constricta* genotypes using SPSS 12 for Windows computer software. A post hoc multiple comparison of the observed means was performed using the LSD test ($p<0.05$). A paired sample t-test was performed to separate means for different parameters between *I. tinctoria* and *I. constricta* at various sowing densities ($p<0.05$).

*Determination of indican percentage*

The indican percentage was determined from fresh leaf samples harvested during the first year and dried in the shade for one week. An indican standard was purchased from the Merck Company.

Each sample consisted of 0.5 g dried leaves pulverized separately and stirred with 10 ml boiling water prior to maceration. This was followed by centrifugation for 5 min at 3000 rpm, and filtration through black band paper with the addition of 10 mm cold water, after which 20 µl of the filtrate was injected into an HPLC column (Strobel and Gröger, 1989; Gilbert et al., 2000). The standard indican peak appeared after 5.5–5.7 minutes (Fig. 1). In addition, the standard indican peak was compared with the Diode Array spectrum.

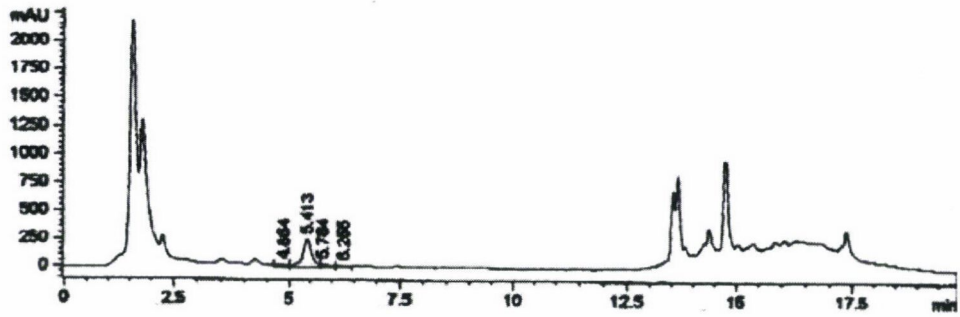



*Fig. 1.* Standard peak showing chromatogram of indican after 5.5–5.7 minutes

To obtain a calibration curve for standard indican, injection was performed three times each for five different solutions prepared in concentrations of 0.00408–0.408 mg $ml^{-1}$. The peak area value obtained was used to calculate the calibration equation (r=0.9999, y=16986.3x – 17.01). Two different extractions were made from each sample and two injections were performed. The percentage of indican in the drug was calculated using the means of the indican peak areas in the calibration equation. In addition, in order to evaluate the accuracy of the trial, recovery tests were performed with a mean recovery of 98.6%.

The indican percentage was determined by HPLC using an HP 1100 chromatograph equipped with a Photodiode Array Detector 1100. The analysis was carried out on a reversed Hypersil C18 column (15 × 4.6, 5 micron, Phenomenex) using $H_2O$–MeOH (20:80) (solvent A) and MeOH (Solvent B) as solvents. The samples were injected into the column through a 20 ml sample loop and the chromatograms were recorded at 280 nm.

## Results and discussion

Phenological data on the *I. tinctoria* and *I. constricta* species are shown in Table 2. The results showed that the sowing density affected various agronomic characteristics of the two genotypes, while variability was also observed between the two genotypes for many characters. Furthermore, *I. tinctoria* started flowering earlier than *I. constricta* and was ready for harvest approximately one week earlier. This may be due to genetic differences between the two species.

*Table 2*
Phenological observations on *Isatis tinctoria* and *Isatis constricta*

| Phenological observations | *I. tinctoria* | *I. constricta* |
|---|---|---|
| Sowing date | 16 Feb. 1999 | 16 Feb. 1999 |
| No. of days to emergence | 12 | 14 |
| No. of days to stalk formation | 402 | 396 |
| No. of days from sowing to 50% flowering | 431 | 424 |
| No. of days from stalk formation to 50% flowering | 30 | 27 |
| No. of days from 50% flowering to fruit formation | 31 | 27 |
| No. of days from 50% flowering to harvest | 41 | 47 |
| No. of days from sowing to harvest | 471 | 470 |

The statistical results showed that both the sowing densities and the genotypes led to sharp variations in the number of leaves per plant, leaf length, petiole length, fresh and dry leaf yield, stem diameter, fruit yield, 1000-fruit weight and 1000–seed weight. The sowing density did not affect leaf width, plant height, first branching height, fruit length or fruit width. However, these characteristics differed for the two genotypes (Tables 3 and 4).

*Table 3*

Means of No. of leaves/plant, leaf length (cm), leaf width (cm), petiole length (cm), fresh leaf production (kg 10 $m^{-2}$), dry leaf production (kg 10 $m^{-2}$), plant height (cm) and stem diameter (mm) of *Isatis tinctoria* and *Isatis constricta* at different sowing densities

| Parameters | 40 × 10 cm | | 40 × 20 cm | | 60 × 10 cm | | 60 × 20 cm | |
|---|---|---|---|---|---|---|---|---|
| | *I. tinct.* | *I. const.* | *I. tinct.* | *I. const.* | *I. tinct.* | *I. const.* | *I. tinct.* | *I. const.* |
| No. of leaves/plant | 12.85cA | 13.08abA | 13.53bA | 12.63bB | 13.55bA | 12.63bB | 16.68aA | 13.78aB |
| Leaf length | 14.03abA | 10.66bB | 13.27bA | 11.23aB | 15.22aA | 10.63bB | 14.84aA | 10.93aB |
| Leaf width | 4.12B | 4.23A | 4.04B | 4.45A | 4.20A | 4.12A | 4.36A | 4.32A |
| Petiole length | 4.03abB | 4.28bA | 3.73bB | 4.25bA | 4.41aA | 4.28bA | 4.13aB | 5.01aA |
| Fresh leaf production | 8.03aA | 7.40aB | 6.56bB | 7.18aA | 6.03bA | 4.44bB | 5.77bA | 4.51bB |
| Dry leaf production | 1.68aA | 1.32aB | 1.31bA | 1.31aA | 1.25bA | 0.79bB | 1.14bA | 0.79bB |
| Plant height | 119.33A | 78.85B | 116.50A | 76.70B | 118.88A | 80.58B | 117.10A | 76.95B |
| Stem diameter | 7.63bB | 9.48bA | 7.75bB | 9.75bA | 7.93bB | 10.18aA | 8.33aB | 10.53aA |

Values within rows followed by different small letters are significantly different at the 0.05 level according to Duncan's test; values within columns followed by different capital letters are significantly different at the 0.05 level according to the t-test.

*Table 4*

Means of first branching height (cm), fruit yield (kg 10 $m^{-2}$), fruit length (cm), fruit width (mm), 1000-fruit weight (g), 1000-seed weight (g), hull content (%), indican percentage (%) and indican yield (g 10 $m^{-2}$) of *Isatis tinctoria* and *Isatis constricta* at different sowing densities

| Parameters | 40 × 10 cm | | 40 × 20 cm | | 60 × 10 cm | | 60 × 20 cm | |
|---|---|---|---|---|---|---|---|---|
| | *I. tinct.* | *I. const.* | *I. tinct.* | *I. const.* | *I. tinct.* | *I. const.* | *I. tinct.* | *I. const.* |
| First branching height | 81.08A | 40.58B | 77.55A | 38.38B | 78.48A | 38.85B | 79.43A | 38.25B |
| Fruit yield | 2.61aB | 3.35aA | 2.92aB | 3.45aA | 2.11bB | 2.63bA | 2.12bB | 2.56bA |
| Fruit length | 2.82A | 2.95A | 2.89B | 3.15A | 2.73B | 3.16A | 2.47B | 3.03A |
| Fruit width | 1.45A | 1.51A | 1.44B | 1.57A | 1.44B | 1.69A | 1.33B | 1.59A |
| 1000-fruit weight | 4.16B | 6.72bA | 4.17B | 6.61bA | 4.47B | 7.62aA | 4.02B | 6.79bA |
| 1000-seed weight | 1.20bB | 1.82bA | 1.19bB | 1.79bA | 1.46aB | 2.04aA | 1.25bB | 1.83bA |
| Hull content | 71.00aA | 72.43A | 71.18aA | 72.95A | 66.73bB | 73.25A | 68.05bB | 73.05A |
| Indican percentage | 0.32bB | 0.41aA | 0.31bB | 0.39bA | 0.39bA | 0.39bA | 0.34aA | 0.38bB |
| Indican yield | 5.70aA | 5.10aB | 4.89abA | 4.17abB | 4.40bA | 4.02abB | 3.65cA | 3.35cB |

Values within rows followed by different small letters are significantly different at the 0.05 level according to Duncan's test; values within columns followed by different capital letters are significantly different at the 0.05 level according to the t-test.

The number of leaves per plant varied between 12.85 and 16.68 in *I. tinctoria* and 12.63 and 13.78 in *I. constricta.* The maximum number of leaves per plant in both genotypes was recorded at a planting density of 60 × 20 cm. The number of leaves per plant is the most important character for dyestuff yield. It is concluded that wide row (60 cm) and plant (20 cm) spacings affected plant development positively due to higher uptake of nutrients and water and better illumination. The number of leaves obtained in this study for *I. tinctoria* was compatible with the figure of 8.76–17.47 leaves reported by Tansı (1999).

Leaf length ranged from 13.27 to 14.84 cm in *I. tinctoria* and from 10.63 to 11.23 cm in *I. constricta.* The results showed that *I. tinctoria* had longer leaves compared to *I. constricta.* The longest leaves in *I. tinctoria* were observed at a planting density of 60 × 20 cm and in *I. constricta* at a planting density of 40 × 20 cm. It is concluded that wide rows (60 cm) and plant spacings (20 cm) affected the number of leaves positively in *I. tinctoria,* while narrow rows (40 cm) and wide plant spacings (20 cm) had a positive effect on the development of *I. constricta* leaves. Hurry (1930) reported that the leaf length of *I. tinctoria* reached as much as 30 cm in rich soils. Yıldırımlı (1988) reported that the leaf length of *I. tinctoria* varied from 6–27 cm and 1.3–13 cm in the middle portions of the stem, while Tansı (1999) reported that the leaf length of *I. tinctoria* varied between 8.51 and 17.24 cm. The results of the present study are compatible with these findings.

Neither sowing density nor genotype caused any distinct difference in the leaf width. However, *I. constricta* tended to have greater leaf width at all sowing densities. Hurry (1930) reported that the leaf width increased to 15 cm in soils rich in nutrients. Yıldırımlı (1988) reported that the basal leaf width of *I. tinctoria* varied between 1 and 3 cm and the leaf width in the middle of the stem varied between 0.4 and 3.0 cm, whereas Tansı (1999) reported that the leaf width of *I. tinctoria* had a range of 1.70 to 4.01 cm.

The petiole length ranged from 3.73 to 4.41 cm in *I. tinctoria* and from 4.25 to 5.01 cm in *I. constricta.* The longest petioles in *I. tinctoria* were observed at a planting density of 60 × 10 cm and in *I. constricta* at a planting density of 60 × 20 cm, showing that wide rows needed to be combined with a plant to plant spacing of 10 cm in *I. tinctoria* and 20 cm in *I. constricta* to give longer petioles. *I. constricta* had longer petioles compared to *I. tinctoria.* Variation in the petiole length was due to genetic differences between the two species and was affected by the row to row and plant to plant spacings. Petiole length had a direct relationship with sowing density, increasing with increased sowing density (Table 3). These observations support the findings of Mısırdalı (1985).

Generally, the first year leaves of woad are preferred for obtaining dye. Branching and flowering, which start in the last week of April or the first week of May during the second year, have a negative effect on the quality and content of indigo. The fresh leaf yield of *I. tinctoria* and *I. constricta* varied from 5.77

(5770 kg $ha^{-1}$) to 8.03 kg 10 $m^{-2}$ (8030 kg $ha^{-1}$) and from 4.51 (4510 kg $ha^{-1}$) to 7.40 kg 10 $m^{-2}$ (7400 kg $ha^{-1}$), respectively. There were significant differences between the plant densities, with narrow row spacings producing more leaf yield than wider row spacings. The fresh leaf yield of *I. tinctoria* was higher than that of *I. constricta*. Garcia and Biertümpfel (1997) reported that the leaf yield of *I. tinctoria* may reach approximately 20,000 kg $ha^{-1}$. However, researchers also emphasise that *I. tinctoria* could be harvested three times a year. In the present study, one harvest was made.

Similarly the dry leaf yield of *I. tinctoria* and *I. constricta* varied from 1.14 to 1.68 kg 10 $m^{-2}$ and from 1.32 to 7.93 kg 10 $m^{-2}$, respectively. The dry leaf yield decreased when the plant density increased. *I. tinctroria* produced a higher dry leaf yield than *I. constricta*. As the leaf length of *I. tinctoria* was greater than that of *I. constricta*, it influenced the dry leaf yield as well. Hurry (1930) and Garcia and Biertümpfel (1997) reported that the dry leaf yield of *I. tinctoria* was 1000–3000 kg $ha^{-1}$ and 3000–4000 kg $ha^{-1}$, respectively, sometimes rising to 5000–7000 kg $ha^{-1}$. The results of the present study are close to the base level results of Hurry (1930).

Sowing density had no effect on the plant height of the respective genotypes. However, *I. tinctoria* plants were considerably taller compared to *I. constricta* at all sowing densities. The tallest plants of *I. tinctoria* showed a plant height of 118.88 cm, whereas those of *I. constricta* only grew to 80.58 cm. This plant height was greater than that reported by Baytop (1984) and Tansı (1999), but lower than that reported by Hurry (1930). The results indicate that the plant height was not affected by external conditions such as soil conditions, irrigation or nutrition, but was affected by the genetic background of the plants.

Stem diameter was affected by both sowing density and genotype. The thickest stems in both genotypes were recorded at the widest row spacing of 60 × 20 cm. In general, the stems of *I. tinctoria* were thinner than those of *I. tinctoria*.

Sowing density did not affect the first branching height of either genotype. However, the first branching height of *I. tinctoria* was higher than that of *I. constricta* at all sowing densities. *I. tinctoria* started branching at a height of 77.55 to 81.08 cm and *I. constricta* at a height of 38.25 to 40.58 cm at all sowing densities. The lower branching height in *I. constricta* compared to *I. tinctoria* is attributed to genetic differences between the species.

There was a sharp variation in the fruit yield of the two genotypes, with a maximum yield of 2.92 kg 10 $m^{-2}$ (2920 kg $ha^{-1}$) in *I. tinctoria* and 3.45 kg 10 $m^{-2}$ (3450 kg $ha^{-1}$) in *I. constricta*. By contrast, Hurry (1930) reported that *I. tinctoria* would give 1270–1905 kg $ha^{-1}$ fruit yield in soils rich in nutrients.

Fruit length and width showed no variation at any sowing density for either genotype. However, they varied between the genotypes. Longer (2.95–3.16 mm) and wider (1.51–1.69 mm) fruits were obtained in *I. constricta*

compared to *I. tinctoria*. This is in contradiction to Hurry (1930), who reported the fruit length of *I. tinctoria* as 4.2 mm, but is in agreement with Yıldırımlı (1988) and Tansı (1999), who reported fruit lengths of 1.5–4 mm and 2.7–3.2 mm, respectively. The results are also in agreement with those of Davis (1965) and Mısırdalı (1985), who reported the fruit width of *I. constricta* as 3–4 and 3–7 mm, respectively.

The 1000-fruit weight of *I. tinctoria* remained unchanged at all sowing densities. However, the sowing density affected the 1000-fruit weight of *I. constricta,* the highest weight being recorded for the 60 × 10 cm sowing density. The 1000-fruit weight of *I. constricta* was considerably higher than that of *I. tinctoria*. The 1000-seed weight in *I. tinctoria* was affected by the genotype and in *I. constricta* by both genotype and sowing density. The seeds of *I. constricta* were heavier than those of *I. tinctoria*. Both genotypes showed the highest seed weight at the 60 × 10 cm sowing density. These findings are in agreement with those of Dolya et al. (1972) and Wurl (1997), who reported the 1000-seed weight of *I. tinctoria* as 1.66 g and 2.00 g, respectively. The 1000-seed weights found in the present work were higher than those reported by Tansı (1999) for *I. tinctoria*. Thousand-fruit and seed weights are very changeable and are affected by various factors such as genotype, cultivation and environmental conditions.

The hull content of *I. tinctoria* and *I. constricta* was not significantly affected by plant density, though narrow spacings tended to result in higher hull content in *I. tinctoria* and wide spacings in *I. tinctoria*. In general, *I. constricta* had higher hull content than *I. tinctoria*.

Like essential oils or tanning agents, plant dyes are secondary plant metabolites (Hartl and Vogl, 2003), and the dyestuff content is an essential criterion for quality. Indican is present in the leaves of *Isatis* species. The indican percentage in both genotypes remained unaffected by the sowing density. However, it varied between the two genotypes with a considerably higher percentage (0.38–0.41%) in *I. constricta.* The highest indican yield was obtained at a sowing density of 40 × 10 cm in *I. constricta* and 60 × 20 cm in *I. tinctoria*. The percentage of indigo in the dry mass is very small (Anonymous, 1997). Strobel and Gröger (1989) reported that the indigo precursor (isatan-B) percentage of *I. tinctoria* varied from 0.05–0.14%, while Anonymous (1997) reported the dyestuff content of *I. tinctoria* as 0.1–1.2%. The present results are higher than those reported by Strobel and Gröger (1989).

The indican yield was significantly affected by plant density and genotype ($p<0.05$). The highest indican yield was obtained using narrow spacing (40 × 10 cm) in both genotypes. The dyestuff yield decreased gradually with wider spacings. The number of plants per unit area is important in order to obtain a greater dyestuff yield.

## Conclusions

The study determines the effects of various sowing densities on the agronomic characteristics of two *Isatis* species. It is clear from the results (Tables 3 and 4) that the maximum number of leaves per plant, leaf length, leaf width, petiole length, stem diameter, fruit length, 1000-fruit weight and 1000-seed weight were obtained with wider spacings of 60 × 10 or 60 × 20 cm. However, for fresh and dry leaf yield 10 $m^{-2}$, plant height, fruit yield, fruit length, low hull content, indican percentage and indican yield, narrow row spacing with a greater plant density was optimal. Thus, it can be concluded that smaller plant densities might be better for obtaining higher indican yield. This information could be helpful for the economical cultivation of these plants to achieve higher indican percentage and yield.

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*Short communication*

# ESTIMATES OF VARIATION AND HERITABILITY OF SOME QUANTITATIVE AND QUALITY CHARACTERS IN *Triticum turgidum* L. ssp. *durum* (Desf.)

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The results obtained for the parental, $F_1$ and $F_2$ generations of a 10 × 10 diallel set (excluding reciprocals) of durum wheat revealed that there were significant differences between all the hundred genotypes for all the characters. The genotypes represented a wide range of expression for almost all the characters. High estimates of GCV (genetic coefficient of variation) were observed for the number of effective tillers, grain yield per plant, harvest index and 1000-grain weight. The low values of GCV recorded for days to heading, grain protein content and plant height indicated their limited scope for improvement. High heritability ($h^2$) values ranging from 92.27% (grain yield/plant) to 99.00% (protein content) were observed for all the characters. The highest expected GA (genetic advance) as a percentage of the mean was manifested for harvest index, followed by plant height, number of effective tillers per plant and grain yield per plant. These traits also possessed high estimates of heritability, indicating that most of the variation in these characters was due to additive gene effects. For protein content high heritability was observed with low genetic gain, indicating non-additive gene effects. Thus, a systematic approach based on selection for harvest index, plant height and number of effective tillers per plant on the basis of high *per se* performance would be the most effective approach for improving the yield level of durum wheat. The wealth of variability available in the hybrid populations offers good prospects for its improvement in the near future.

**Key words:** durum wheat, genotypes, genetic coefficient of variation, heritability, genetic advance, grain yield, yield components, gene effects

## Introduction

Durum is the second most important cultivated species of the genus *Triticum* in India, occupying about 2.5 million hectares. Despite its importance for the human diet, little progress has been made in improving the yield and nutritional qualities in the country as compared to bread wheat. Historically, durum wheat has received insufficient attention from plant breeders. In recent years, the introduction of dwarfing genes and rust resistance have created

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renewed interest in extending durum wheat cultivation in India, particularly under high input management conditions in northern parts. Therefore, systematic attempts to improve durum are needed through the manipulation of various yield components.

Grain yield is a function of accumulated carbon, manifested as the maximum number of tillers with the widest possible leaves, the maximum number of spikelets per head, the maximum number of florets per spikelet, and the plumpest possible kernels in each floret. It is well documented that yield is a very complex character, direct selection for which is not effective. Early breeders (Watson, 1952; Stoskopf and Reinbergs, 1965; Adams, 1967) suggested selection for yield components as a possible method for yield improvement. The presence of genetic variability in the base population is an essential requirement for achieving success in a selection programme. Very little information is available on the relative importance of the component characters of grain yield in durum wheat. Quantitative characters are greatly influenced by environmental factors, which necessitates knowledge of the variability owing to genetic factors, the actual genetic variation heritable in the offspring and the advance which can be made through the selection of superior individuals. The harvest index, which denotes the partitioning of photosynthates between productive and non-productive parts, is also important for the grain yield (Gill et al., 1980; Kulshrestha and Jain, 1982; Balla et al., 1986; Dhindsa and Bains 1987; Menon and Sharma, 1993; Sharma and Sharma, 1996). Hence it was also included in the present study, which was undertaken to investigate the genotypic coefficient of variation, heritability and genetic advance for grain yield and its component traits in order to give the durum wheat breeder some idea of the behaviour of these attributes and of how to use them while planning for the improvement of durum wheat.

## Materials and methods

The experimental material consisted of the parental, $F_1$ and $F_2$ generations of a 10 × 10 diallel set (excluding reciprocals) in durum wheat. The parents were selected on the basis of existing variability for most of the quantitative characters and protein content. Ten genotypes of durum wheat, namely PBW 34, PDW 215, PDW 233, PDW 236, WH 896, Raj 1555, Raj 911, Cocorit 71, HI 8062 and A-9-30-1, were included. The parents, along with their 45 $F_1$ and 45 $F_2$ generations, were planted in a randomized block design with three replications at the Agricultural Research Station, Durgapura, Jaipur, Rajasthan, India. Each row was 5 m long with a spacing of 30 cm between rows and 10 cm between plants. Each parent and $F_1$ was planted in two rows and each $F_2$ in six rows. Observations were recorded on 20 randomly selected plants for the parents and $F_1$s and on 60 plants in the $F_2$ families for days to flowering (75%), plant height (cm), number of effective tillers per plant, 100-kernel weight (g), grain yield per plant (g), harvest index (%) and protein content (%). The nitrogen content of the grains was estimated by the standard micro-Kjeldahl method. Protein content was determined by multiplying the Kjeldahl nitrogen by a factor of 6.25. Using the mean values, the data were analysed for variation following the standard statistical procedures suggested by Panse and Sukhatme (1967). The genetic coefficient of variation (GCV), heritability in the broad sense ($h^2$) and genetic advance (GA) were calculated according to Burton (1952), Panse and Sukhatme (1967) and Johnson et al. (1955), respectively.

## Results and discussion

The results of the present study revealed that there were significant differences between all the hundred genotypes for all the characters. The genotypes represented a wide range of expression for almost all the characters (Table 1). High estimates of GCV were observed for the number of effective tillers, grain yield per plant, harvest index and 100-kernel weight. The low values of GCV recorded for days to heading, grain protein content and plant height indicated their limited scope for improvement. The estimation of GCV alone does not indicate the amount of heritable variation. Burton (1952) suggested that GCV computed along with heritability estimates would give a better picture for selection on the basis of phenotypic performance. In the present study high heritability ($h^2$) values ranging from 92.27% (grain yield/plant) to 99.00% (protein content) were observed for all the characters. Tripathi et al. (1973), Maloo (1984), Sharma and Kaul (1986), Maloo et al. (1993) and Tiwari et al. (1993) also reported similar results in durum wheat.

Johnson et al. (1955) reported that heritability estimates along with genetic advance are more useful than heritability alone in the prediction of the resultant effect for selecting the best individual genotype. The highest expected GA as a percentage of the mean was recorded for the harvest index, followed by plant height, number of effective tillers per plant and grain yield per plant (Table 1). These traits also possessed high estimates of heritability, indicating that most of the variation in these characters was due to additive gene effects. Sharma and Singh (1983), Nass and Jui (1985), Sharma and Kaul (1986), Raghuvanshi et al. (1988) and Patil et al. (1997) also observed additive gene effects in wheat. High heritability combined with low genetic gain was observed for protein content, indicating non-additive gene effects. Ram and Srivastava (1974) and Zittelli et al. (1978) also observed high heritability for protein content. Thus, a systematic approach to yield, involving selection for harvest index, plant height and number of effective tillers per plant on the basis of high *per se* performance, would be most effective for the improvement of yield levels in durum wheat. The wealth of variability available in hybrid populations offers good prospects for its improvement in the near future.

*Table 1*
Genetic parameters for different characters of durum wheat

| Character | Range | Mean | S.E. (±) | Genetic coefficient of variation | Heritability (%) | Genetic advance as percentage of mean |
|---|---|---|---|---|---|---|
| Days to heading | 67.13–82.13 | 74.42 | 0.62 | 4.38 | 94.69 | 6.53 |
| Plant height | 69.50–88.75 | 79.54 | 0.93 | 8.68 | 95.58 | 10.69 |
| No. of effective tillers per plant | 6.25–14.30 | 9.43 | 0.41 | 19.57 | 92.98 | 8.66 |
| 100-kernel weight | 4.20–6.60 | 5.25 | 0.12 | 13.47 | 95.50 | 1.42 |
| Grain yield per plant | 13.30–31.40 | 21.20 | 1.01 | 18.48 | 92.27 | 7.75 |
| Harvest index | 33.04–56.78 | 44.10 | 1.16 | 13.71 | 94.73 | 12.18 |
| Protein content | 10.80–13.86 | 12.56 | 0.07 | 7.81 | 99.00 | 2.01 |

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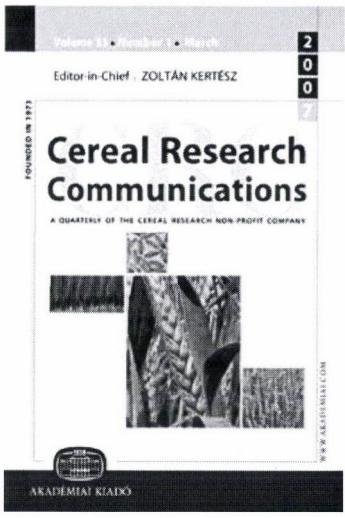
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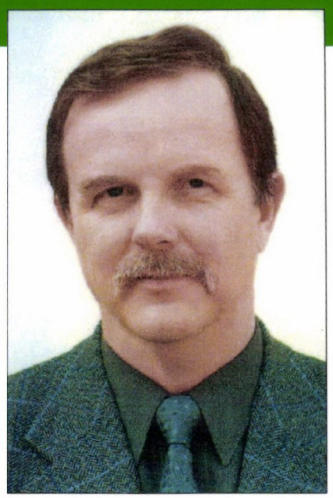
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# CONTENTS

ORIGINAL PAPERS

Rutin content of the grain of buckwheat (*Fagopyrum esculentum* Moench. and *Fagopyrum tataricum* Gaertn.) varieties grown in southern Italy
*A. Brunori* and *G. Végvári* ..... 265

*In vitro* and *in vivo* screening for cold tolerance in winter × spring wheat-derived doubled haploids following the wheat × maize system
*S. Sharma* and *H. K. Chaudhary* ..... 273

Protective effect of two synthetic compounds against chlorsulfuron injury in maize (*Zea mays* L.)
*G. Stoilkova* and *P. Yonova* ..... 283

Properties of humic and fulvic acids under the long-term use of fertilizers in a rice-wheat-cowpea system on a Mollisol
*D. K. Das* and *Nand Ram* ..... 293

Gene system governing stomatal number and specific leaf weight in bread wheat (*Triticum aestivum* L.)
*A. Kumar* and *S. C. Sharma* ..... 299

Evaluation of blue-green algal inoculation on specific soil parameters
*K. Swarnalakshmi, D. W. Dhar* and *P. K. Singh* ..... 307

Co-inoculation of nitrogen-fixing and phosphate-solubilizing bacteria to promote growth, yield and nutrient uptake in chickpea
*P. A. Wani, M. S. Khan* and *A. Zaidi* ..... 315

Performance and gene effects for root characters and micronutrient uptake in wheat inoculated with arbuscular mycorrhizal fungi and *Azotobacter chroococcum*
*R. Singh, R. K. Behl, P. Jain, N. Narula* and *K. P. Singh* ..... 325

Assessment of genotype × environment interactions for yield and morphine content in opium poppy (*Papaver somniferum* L.)
*H. K. Yadav, S. Shukla* and *S. P. Singh* ..... 331

Correlations and path analysis of yield components in synthetic varieties of sunflower (*Helianthus annuus* L.)
*A. T. Göksoy* and *Z. M. Turan* ..... 339

Effect of residue recycling on a cropping system based on fodder sorghum
*E. Shekinah, A. Alagesan, C. Jayanthi* and *N. Sankaran* ..... 347

Effects of post-emergent herbicides on *Trichoderma harzianum,* a potential biocontrol agent against *Sclerotinia sclerotiorum* in soybean cropping
*M. C. Rollán, C. Mónaco, G. Lampugnani, N. Arteta, D. Bayo* and *M. I. Urrutia* .... 355

Morphological attributes of oriental spruce [*Picea orientalis* (L.) Link.] seedlings grown in peat-based media amended with natural zeolite
*S. Ayan* and *F. Tilki* ..... 363

RAPD variation of late-maturing sorghum [*Sorghum bicolor* (L.) Moench] landraces from Ethiopia
*S. Mamo, A. Ayana* and *T. Tesso* ..... 375

Productivity and nitrogen use efficiency parameters in cotton cultivars with varying N levels and soil types under rainfed conditions
*M. V. Venugopalan, K. B. Hebbar, P. Tiwary, S. Chatterji, V. Ramamurthy, O. Challa* and *B. A. Sonune* ..... 383

OBITUARY ..... 393

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# RUTIN CONTENT OF THE GRAIN OF BUCKWHEAT (*Fagopyrum esculentum* Moench. and *Fagopyrum tataricum* Gaertn.) VARIETIES GROWN IN SOUTHERN ITALY

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The rutin content of the grain of 31 buckwheat varieties (28 *F. esculentum* and 3 *F. tataricum*) grown on the high plain of Sila (Region of Calabria) and on the massif of Pollino (Region of Basilicata) presented a substantial degree of variation.

Among the *F. esculentum* varieties the lowest values were found for Botan and Spacinska, while the highest contents were observed in the varieties Emka and Lechnicka Krajova. As expected, the rutin content of the grain of *F. tataricum* was higher than that of *F. esculentum*, with the variety Donan showing the highest value.

In view of the role of rutin in conferring most of the functional food characteristics of buckwheat, knowledge of the grain rutin content expressed in a specific environment, together with the yield potential, is important for the identification of buckwheat varieties worth adopting in an area such as the high plain of Sila, which, thanks to its extent, has the potential to represent a novel territory for the profitable cultivation of buckwheat in Italy.

**Key words**: buckwheat, grain rutin content, variety evaluation

## Introduction

The flavonoid rutin is a glycoside of the flavonol quercetin. The glycoside portion of rutin is the disaccharide rutinose (rhamnose and glucose).

Rutin is the active compound responsible for most of the beneficial health properties credited to the consumption of buckwheat grain. Besides the widely recognised positive effect on capillary fragility and permeability, rutin is reported to have an antihyperglycaemic effect (Wang et al., 1992; Kamalakkannan and Stanely Mainzen, 2006), a protective effect against the development of diabetes (Odetti et al., 1990; Srinivasan et al., 2005), a mitigating effect on the consequences of diabetes (Je et al., 2002; Nagasawa et al., 2003), antioxidative properties (Oomah and Maza, 1996; Afanas'eva et al., 2001), anti-inflammatory activity (Guardia et al., 2001), anti-platelet formation properties (Sheu et al., 2004), a mitigating effect on cardiovascular diseases (He

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et al., 1995), anticancer activity (Park and Park, 2004) and antimutagenic activity (Aheme and O'Brien, 1999; 2000; Undeger et al., 2004).

Although rutin is present in much higher concentrations in the leaves and in particular in the flower (up to 3%) (Fabjan et al., 2003), thus representing convenient sources of rutin for medicinal purposes, enough rutin is found in the grain to confer food containing buckwheat flour the characteristics of FOSHU (Foods for Specific Health Use).

Apart from food for people affected by celiac disease, where buckwheat alone is to be preferred because of the absence of gluten, preparations containing various proportions of buckwheat are currently advertised as salutary food in Italy.

In this respect high rutin content in the grain is a bonus for both bakeries and the agroindustry, in that either a lower proportion of buckwheat can be used or a higher rutin level can be claimed. There thus seems to be scope for improving buckwheat production by selecting for high grain rutin content in the top-yielding varieties suited for a given environment.

Variations were reported in the rutin content of the grain of buckwheat varieties (Kitabayashi et al., 1995a; Ohsawa and Tsutsumi, 1995; Oomah and Mazza, 1996). In addition, the content of rutin was found to be influenced by location (Oomah and Mazza, 1996) and flowering time, increasing under long-day conditions (Ohsawa and Tsutsumi, 1995). With regard to the species, *F. tataricum* has a much higher grain rutin content than *F. esculentum* (Kitabayashi et al., 1995b; Fabjan et al., 2003; Park et al., 2004). However, *F. tataricum* is a lower yielder compared to *F. esculentum* (Fabjan et al., 2003).

The recent agronomic assessment of the yield potential of buckwheat varieties, carried out in the summer of 2004 and 2005 at a number of sites in Central and Southern Italy new to the cultivation of buckwheat, showed quite appreciable levels of grain yield. Furthermore, at each site different varieties turned out to be top yielders (Brunori et al., 2005; 2006).

The level of grain yield observed in 2004 and 2005, approaching 2.0 t/ha (Brunori et al., 2005; 2006), indicated that the cultivation of buckwheat could be economic in Southern Italy. However, the large variation in grain yield observed between the varieties emphasizes the need to run preliminary assessments of variety adaptation to specific areas. Due to its relevance for quality, a knowledge of the rutin content of the grain may further contribute to the identification of the best varieties among those which proved to be good grain yielders.

For this purpose, the grains of *F. esculentum* varieties, grown in the summer of 2005 on the high plain of Sila in the Region of Calabria, were analysed for rutin content. Due to its extent the high plain of Sila could represent a novel area for large-scale buckwheat cultivation. Although none of the three varieties of *F. tataricum* under investigation gave an appreciable grain yield in the Sila environment, they performed quite satisfactorily on the massif of Pollino, an important mountain area of the Basilicata Region. Because of the high rutin content expected in the grain of *F. tataricum*, these varieties were included in the present investigation.

## Materials and methods

*Fagopyrum esculentum* varieties were grown in the summer of 2005 at Camigliatello Silano on the high plain of Sila in the Region of Calabria (Southern Italy).

Grain samples were obtained from both an agronomic trial for variety assessment and from seed multiplication plots. In addition, three *F. tataricum* varieties grown on the nearby massif of Pollino in the Region of Basilicata, two of which proved to be good yielders, were included.

The grains of the following varieties were analysed:

– *F. esculentum* varieties from an agronomic trial in Camigliatello Silano: AC Manisoba, Bamby, Darja, Emka, Jana, Kitawasesoba, Koban, Kora, Lileja, Luba, Mancan, Panda, Pyra, Spacinska and Springfield;

– *F. esculentum* varieties from seed multiplications at the same location: Aelita, Anita Belorusskaya, Arakawa Village, Bolshevik 4, Botan, Česka Krajova, Hruszowska, Iliya, Kara-dag, Lechnicka Krajova, Lena, Prego and Svityazyanka;

– *F. tataricum* varieties from agronomic trials on the massif of Pollino: Donan, Golden and Ishisoba.

The analysis of rutin content was carried out in the HPLC laboratory of Corvinus University, Budapest. Wholemeal was prepared from the grain with the use of a Foss Tecator Cyclotec 1093 sample mill. Samples weighing 1 g each were extracted with 10 ml methanol for 24 hours at room temperature in the dark. Due to the expected higher content of rutin, the volume of methanol was doubled (20 ml) in the case of *F. tataricum* varieties. At the end of the extraction period the vials were placed for 5 minutes in an Elma Transsonic T420 ultrasonic bath operating at a frequency of 35 kHz. The methanol fraction was recovered by centrifuging for 5 min at 15,000 rpm with a centrifuge (Hettich Mikro 22R model). Before analysis the methanol fraction was filtered by passing through a Millex HN 0.45 syringe-driven filter unit.

The rutin content was determined with a Waters HPLC equipped with a plus auto-sampler, a binary HPLC pump, and a dual absorbance detector operating at 350 nm wavelength. The active content was separated on a Symmetry $C_{18}$ 5 µm 4.6×150 column. The analytical process was assisted by Empower $^{2}$TM software. The analysing conditions were: 2.5% acetic acid in water (350 ml), MeOH (50 ml), acetonitrile (100 ml) as mobile phase; flow-rate was 1 ml $min^{-1}$; pressure on the column was 1750 ± 10 psi. Twenty µl of seed extract in methanol was utilized for the rutin determination. Two replicated analyses were run for each sample. Reference was made to a solution of rutin hydrate [153-18-4] containing 2.65 mg dissolved in 25 ml of methanol (HPLC quality).

## Results

An appreciable variation in the rutin content of the grains was apparent among varieties of *F. esculentum*, ranging from 9.0 mg/100 g dry weight (DW) for the variety Botan and 9.2 mg/100 g DW for Spacinska to 36.2 mg/100 g DW for Emka (Table 1). The expected superior content of rutin in the grain of *F. tataricum* was clearly evident on the chromatograms (Fig. 1), the highest average value being recorded for the variety Donan (2,132.1 mg/100 g DW ) (Table 1).

The rutin content of *F. esculentum* varieties grown for agronomic assessment at Camigliatello Silano is presented together with grain yield data as reported by Brunori et al. (2006) in Table 2. Four of the six top-yielding varieties (1.6–1.8 t/ha), Kora, Jana, Lileja and Luba, had satisfactory levels of grain rutin content, ranging from 21.7 to 27.3 mg/100 g DW. The *F. tataricum* varieties Donan and Golden, which gave poor yields on the high land of Sila (Brunori et al., 2006), were ranked 1$^{st}$ and 4$^{th}$ on the massif of Pollino, while retaining a high rutin content in the grain (Table 1).

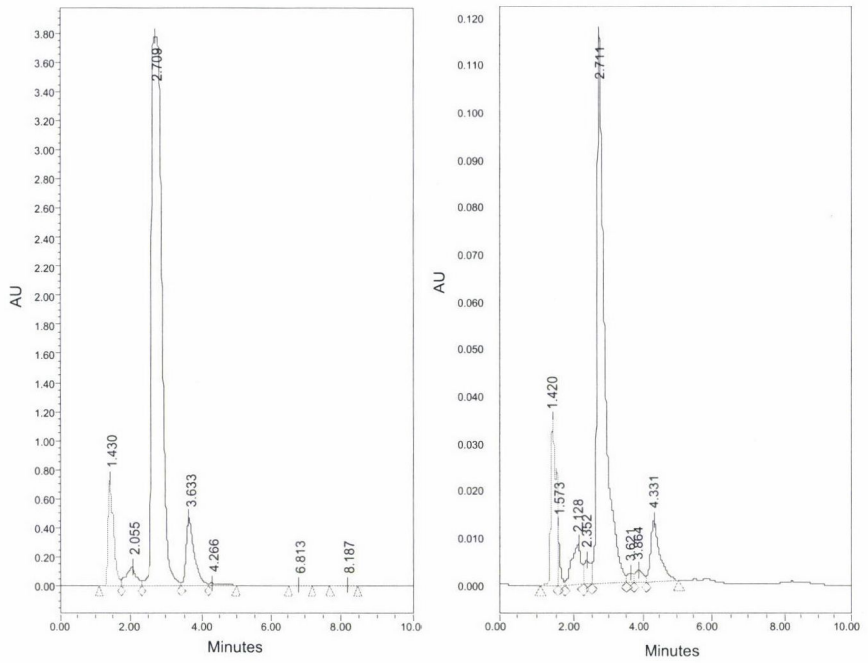



*Fig. 1.* Characteristic HPLC chromatograms for extraction from *Fagopyrum tataricum* (left) and *F. esculentum* (right) varieties, showing the rutin content of the former group to be over 30-fold higher compared to the latter. During chromatography the rutin peak appears after around 2.710 minutes of the running time for the total extract

For the *F. esculentum* varieties under investigation, the grain yield and grain rutin content were not correlated to any appreciable extent ($R^2 = 0.0502$) (Fig. 2).

## Discussion

The substantial variation observed in the rutin content of the grain (Table 1) confirmed previous observations on the variability of this seed component among *F. esculentum* varieties (Ohsawa and Tsutsumi, 1995; Oomah and Mazza, 1996).

As expected, the *F. tataricum* varieties had superior grain rutin content compared to *F. esculentum* (Fabjan et al., 2003).

In view of the relevance of rutin in making the agroindustry interested in buckwheat, *F. tataricum* deserves particular attention for its superior grain rutin content, wherever the environmental conditions enable it to give a good grain yield. This appears to have been the case in Terranova del Pollino in summer 2005, when two *F. tataricum* varieties showed a remarkable grain yield of about 2.0 t/ha, higher than most of the *F. esculentum* varieties (Brunori et al., 2006). High quality buckwheat production can thus be foreseen on the massif of Pollino if the cultivation of *F. tataricum* is adopted.

*Table 1*
Rutin content of buckwheat grain of 28 *F. esculentum* varieties grown at Camigliatello Silano (Region of Calabria) and 3 *F. tataricum* varieties grown at Terranova del Pollino (Region of Basilicata) in 2005

| Varieties | Rutin (mg/100 g dry weight) | | | |
|---|---|---|---|---|
| | Replicated analysis | | Average | ST. DEV. |
| | 1st | 2nd | | |
| *F. esculentum* | | | | |
| AC Manisoba | 17.8 | 15.7 | 16.8 | 1.52 |
| Aelita | 11.8 | 11.5 | 11.6 | 0.21 |
| Anita Belorusskaya | 19.8 | 18.6 | 19.2 | 0.89 |
| Arakawa Village | 12.5 | 11.6 | 12.1 | 0.65 |
| Bambi | 9.2 | 10.1 | 9.7 | 0.60 |
| Bolshevik 4 | 19.2 | 17.7 | 18.5 | 1.05 |
| Botan | 9.6 | 8.3 | 9.0 | 0.93 |
| Česka Krajova | 30.4 | 26.7 | 28.5 | 2.60 |
| Darja | 9.4 | 10.0 | 9.7 | 0.40 |
| Emka | 38.0 | 34.4 | 36.2 | 2.61 |
| Hruszowszka | 22.4 | 21.6 | 22.0 | 0.52 |
| Iliya | 29.7 | 28.6 | 29.2 | 0.77 |
| Jana | 22.4 | 21.0 | 21.7 | 1.04 |
| Kara Dag | 23.7 | 25.5 | 24.6 | 1.30 |
| Kitawasesoba | 25.1 | 24.7 | 24,9 | 0,27 |
| Koban | 21.5 | 21.4 | 21.4 | 0.04 |
| Kora | 23.6 | 21.8 | 22.7 | 1.32 |
| Lechnicka Krajova | 27.9 | 31.4 | 29.7 | 2.48 |
| Lena | 29.0 | 29.7 | 29.3 | 0.45 |
| Lileja | 25.4 | 29.3 | 27.3 | 2.76 |
| Luba | 22.5 | 23.1 | 22.8 | 0.39 |
| Mancan | 19.9 | 23.2 | 21.6 | 2.30 |
| Panda | 15.2 | 15.2 | 15.2 | 0.00 |
| Prego | 24.7 | 25.7 | 25.2 | 0.70 |
| Pyra | 11.9 | 11.5 | 11.7 | 0.30 |
| Spacinska | 8.7 | 9.7 | 9.2 | 0.72 |
| Springfield | 14.2 | 16.0 | 15.1 | 1.30 |
| Svityazyanka | 28.0 | 30.1 | 29.0 | 1.46 |
| *F. tataricum* | | | | |
| Donan | 2115.7 | 2148.5 | 2132.1 | 23.16 |
| Golden | 2107.9 | 2059.4 | 2083.7 | 34.29 |
| Ishisoba | 1762.1 | 1660.5 | 1711.3 | 71.84 |

ST. DEV. = standard deviation.

In spite of the relevance of both grain yield and rutin content, little is known about the relationship between these two characteristics. In line with previous observations on the lack of correlation between grain yield and rutin content in a group of common buckwheat varieties adapted to short day conditions (Ohsawa and Tsutsumi, 1995), the present data indicate that the two traits are largely independent and that satisfactory grain rutin content can be

*Table 2*
Rutin content and grain yield of 15 *F. esculentum* buckwheat varieties grown at Camigliatello Silano (Region of Calabria) in the summer of 2005, expressed as mg/100 g dry weight (DW) as reported by Brunori et al. (2006)

| Varieties | Rutin content (mg/100 g DW) | ST. DEV. | Grain yield (t/ha) |
|---|---|---|---|
| Kora | 22.7 | 1.32 | 1.8 |
| Jana | 21.7 | 1.04 | 1.7 |
| Lileja | 27.3 | 2.76 | 1.7 |
| Luba | 22.8 | 0.39 | 1.7 |
| Panda | 15.2 | 0.00 | 1.7 |
| Pyra | 11.7 | 0.30 | 1.6 |
| Spacinska | 9.2 | 0.72 | 1.4 |
| Darja | 9.7 | 0.40 | 1.3 |
| Bambi | 9.7 | 0.60 | 1.1 |
| AC Manisoba | 16.8 | 1.52 | 1.0 |
| Kitawasesoba | 24.9 | 0.27 | 1.0 |
| Mancan | 21.6 | 2.30 | 1.0 |
| Koban | 21.4 | 0.04 | 0.8 |
| Springfield | 15.1 | 1.30 | 0.8 |
| Emka | 36.2 | 2.61 | 0.5 |

ST. DEV. = standard deviation.

observed in high-yielding varieties such as Kora, Jana, Lileja and Luba (Table 2). However, it could be that the small number of varieties analysed may have influenced the type of correlation observed.

Even though the present investigation on grain rutin content evidenced appreciable variability among the limited number of varieties evaluated, the effect of environmental conditions on this character (Oomah and Mazza, 1996), which might increase interest in growing buckwheat in specific sites in Southern Italy, remains to be verified.

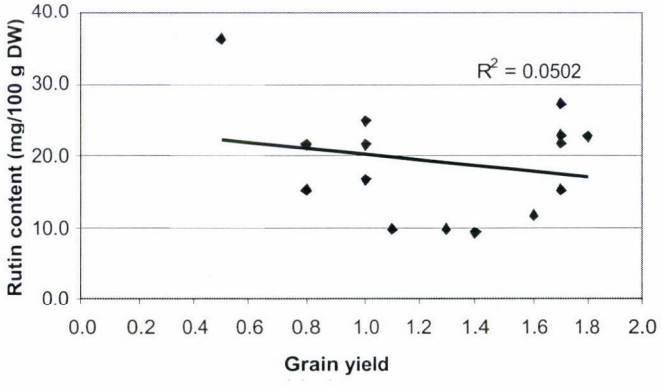



*Fig. 2.* Grain rutin content and grain yield of 15 buckwheat (*F. esculentum*) varieties under agronomic assessment in Camigliatello Silano (Region of Calabria) in summer 2005

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# *IN VITRO* AND *IN VIVO* SCREENING FOR COLD TOLERANCE IN WINTER × SPRING WHEAT-DERIVED DOUBLED HAPLOIDS FOLLOWING THE WHEAT × MAIZE SYSTEM

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Seventy-eight doubled haploid (DH) lines, derived from 21 elite and diverse winter × spring wheat $F_1$ hybrids, following the wheat × maize system, were screened along with the parental genotypes under *in vitro* and *in vivo* conditions for cold tolerance. Under *in vitro* conditions, the 2,3,5-triphenyl tetrazolium chloride (TTC) test was used to characterize the genotypes for cold tolerance. Based on the TTC test, only one doubled haploid, DH 69, was characterized as cold-tolerant, seven DH and five winter wheat parents were moderately tolerant, while the rest were susceptible. Analysis of variance under *in vivo* conditions also indicated the presence of sufficient genetic variability among the genotypes (DH lines + parents) for all the yield-contributing traits under study. The correlation and path analysis studies underlined the importance of indirect selection for tillers per plant, harvest index and grains per spike in order to improve grain yield. It was also concluded that selection should not be practised for grain weight per spike as it would adversely affect the grain yield per plant. When comparing the field performance of the genotypes with the *in vitro* screening parameters, it was concluded that in addition to the TTC test, comprising a single parameter, other physiological and biochemical *in vitro* parameters should be identified, which clearly distinguish between cold-tolerant and susceptible genotypes and also correlate well with their performance under field conditions.

**Key words:** cold tolerance, *in vitro*, *in vivo*, doubled haploids, winter × spring wheat hybridization, wheat × maize system

**Abbreviations:** DH: doubled haploid, TTC: 2,3,5-triphenyl tetrazolium chloride, PCV: phenotypic coefficient of variation, GCV: genotypic coefficient of variation

## Introduction

Low temperature is one of the primary stress factors that limit the growth and productivity of food-grain crops grown in the winter season. The growth and development of wheat plants at different stages is affected by low temperature, leading to reduced yield. Christiansen and St. John (1981) and McWilliam

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(1983) reported that cold-affected plants generally suffer water stress, often due to the chilling of the intact root, which results in wilting. To cope with low temperature stress, one of the most promising breeding approaches is to utilize the gene-pool of winter wheat, which is grown only in the temperate high hills of the north-western Himalayas. Winter wheats are known to carry desirable genes for resistance to cold and can be exploited for the genetic upgrading of the spring wheat cultivars that are predominantly grown. Besides cold hardiness and drought tolerance (Rajaram and Skovmand, 1977), winter wheats can also contribute a well-developed crown root system, better quality, profuse tillering, large and highly fertile square heads, high biomass, enhanced yield and high regenerability to the spring wheats. Further, winter wheats may provide additional sources of resistance to stripe rust, leaf rust and powdery mildew and can be exploited for the amelioration of spring wheats following winter × spring wheat hybridization. Such a breeding approach has the potential to produce a wide range of recombinants tolerant to cold stress.

When screening for crucial and complex traits such as cold tolerance, it would be desirable first to isolate homozygous lines from winter × spring wheat $F_1$ hybrids and then utilize *in vitro* and *in vivo* screening parameters to improve the selection efficiency for cold tolerance. For the rapid isolation of homozygous lines (doubled haploid, DH) from winter × spring wheat $F_1$ hybrids in the shortest possible time, the doubled haploid breeding approach following the wheat × maize system (Laurie and Bennett, 1988) has proved to be of immense significance (Chaudhary et al., 2002; Sharma et al., 2005). Cold tolerance is a complex phenomenon, so the screening of bread wheat for cold tolerance is difficult. Many studies have relied upon natural selection pressures to determine differences in these abilities. However, field survival trials are often inconclusive due either to complete winter-kill or a lack of it (Quisenberry and Clark, 1929; Fowler and Gusta, 1979). For this reason, the investigator must either wait for a winter of the desired severity or distribute material over a wide area in the hope of obtaining the desired stress level at one or more places. However, the use of multi-environment testing is resource expensive and has not improved the reliability of winter hardiness selection (Blum, 1988). Because of the limitations inherent in field trials, there has been a continuing search for rapid, efficient laboratory methods for testing the cold resistance of cultivars. Laboratory procedures to measure freezing tolerance have been developed by a number of investigators. These include changes in luminescence (Brzostowicz and Barcikowska, 1987), plant tissue water content (Brule-Babel and Fowler, 1988), or leakage from plant cells after freezing stress (Teutonico et al., 1993). Meristem regrowth after plants are subjected to freezing temperatures is also commonly used to estimate cold tolerance (Fowler et al., 1981; Andrews and Morrison, 1992). Allard et al. (1998) suggested that the accumulation of the osmolyte betaine is correlated with the development of freezing tolerance in wheat. A greater accumulation of glycine betaine and decreased cell membrane

leakage in frost-acclimated wheat doubled haploid lines has been found to increase cold tolerance and grain yield (Gunawardena et al., 2004). Laboratory screening for cold tolerance has allowed investigators to gather information on cold tolerance that would otherwise be unobtainable in the field. Sethi et al. (1993) advocated screening for cold tolerance under controlled conditions by subjecting the seedlings to artificial freezing for a specific time; genotypes which exhibit recovery after stress can be expected to be cold-tolerant. Moore and Goodsell (1965) used the tetrazolium test to predict the cold tolerance of corn seeds and concluded that it was a rapid, useful method for evaluating embryo soundness and could also serve a useful purpose in predicting and interpreting cold test performance. Therefore, the present investigation was a unique attempt to utilize the tetrazolium test as an *in vitro* screening technique and correlate its results with *in vivo* screening parameters with a view to improving selection efficiency for cold tolerance.

## Materials and methods

In the present investigation, 78 DH lines derived from 21 elite and diverse winter × spring wheat $F_1$ hybrids along with their parental genotypes (Chaudhary et al., 2002; Sharma et al., 2005) were evaluated for cold tolerance under *in vitro* conditions in the Cytogenetics and Tissue Culture Laboratory, Department of Plant Breeding and Genetics, CSK HP Agricultural University, Palampur and under field conditions in the dry temperate region at the High Altitude Research and Extension Centre (HAREC) of CSK HPAU, Kukumseri (Lahaul and Spiti).

*Experiment 1:* In vitro *screening method*

All the DH lines, along with the parental genotypes, were subjected to the triphenyl tetrazolium chloride (TTC, Sisco Research Laboratories Pvt. Ltd., T-829443) test (Kittock and Law, 1968). Seedlings were raised in a sand : soil (1:1) mixture. After 8 days of germination, the seedlings were put in the refrigerator for hardening at 1–4°C for a period of 7 days. The hardened seedlings were then subjected to freezing at a temperature of –9°C in a deep freezer for 24 hours, followed by thawing at 1–4°C for 1 day. Three seedlings of each genotype were then dipped for 5–6 hours in 1% TTC solution in each of the three replications.

In surviving cells the oxidized form of TTC, which was colourless, was reduced to the coloured form (formazan). Formazan was extracted by placing the seedlings in test tubes and covering them with 3 ml methyl cellosolve (2-methoxyethanol). The colorimetric estimation of formazan was done at 480 nm as described by Kittock and Law (1968) and the average optical density of each genotype was calculated for each replication. The optical density of formazan was used to categorize the genotypes as cold-tolerant ($OD_{480}$ = 0.141–0.199), intermediate ($OD_{480}$ = 0.100–0.140) and cold-susceptible ($OD_{480} \leq 0.099$).

*Experiment 2*: In vivo *screening method*

All the 78 doubled haploid lines, along with the parental genotypes, were raised in a random block design at HAREC, Kukumseri (Lahaul and Spiti). Twenty seeds of each genotype were sown in one-metre single rows with inter-row and inter-plant spacings of 23 cm and 5–6 cm, respectively, during October 2003 in three replications.

Data on various grain yield-related traits (effective tillers per plant, spikelets per spike, grains per spike, grain weight per spike, grain yield per plant, 1000-grain weight and harvest index) were recorded on five randomly selected competitive plants from each replication. The data

were analysed using analysis of variance for the experimental design. Basic statistical parameters such as mean, range, coefficient of variation, broad sense heritability ($h^2_{bs}$) and genetic advance were determined. To categorize the magnitude of different parameters, the following limits were used:

| | High | Moderate | Low |
|---|---|---|---|
| Phenotypic coefficient of variation (PCV) | > 20% | 10– 20% | <10% |
| Genotypic coefficient of variation (GCV) | > 20% | 10–20% | <10% |
| Heritability ($h^2_{bs}$) | > 80% | 50– 80% | <50% |
| Genetic advance | > 50% | 30–50% | <30% |

Correlation coefficients (Al-Jibouri et al., 1958) were calculated between the various traits studied. Path coefficient analysis (Dewey and Lu, 1959) was carried out to partition the correlation coefficients of yield components into direct and indirect effects on grain yield.

## Results and discussion

In vitro *screening for cold tolerance*

Analysis of variance showed the existence of enormous genetic variability among the DH lines for this trait. Based on the TTC test, the doubled haploid line DH 69 was identified as cold-tolerant. In addition, DH 22, DH 27, DH 56, DH 65, DH 67, DH 68 and DH 73 amongst the doubled haploids and Saptdhara, Sentry, VWFW 452, VWFW 499 and W 10 amongst the parents were moderately cold-tolerant (Table 1), while the rest of the genotypes were susceptible. Among the doubled haploid lines evaluated under field conditions at HAREC, Kukumseri (Lahaul and Spiti), DH 69 had an average number of spikelets per spike, high grains per spike, high grain weight per spike and high 1000-grain weight. The doubled haploid line DH 22 was characterized by a higher number of spikelets per spike, average number of grains per spike, average grain weight per spike, average harvest index and high 1000-grain weight. DH 27, DH 56, DH 65 and DH 68 had high 1000-grain weight, average grain weight per spike and an average number of spikelets per spike. DH 67 and DH 73 were characterized by an average number of tillers per plant, spikelets per spike and 1000-grain weight.

In vivo *screening for cold tolerance*

At HAREC, Kukumseri (Lahaul and Spiti), the crop received a total rainfall of 226.9 mm with minimum and maximum temperatures ranging from –14.8°C to –16.5°C and –5.13° C to 29.87°C, respectively, during the crop season (Oct. 2003 to Aug. 2004). Analysis of variance indicated the presence of sufficient genetic variability among the genotypes (DH lines + parents) for all the yield-contributing traits under study (Table 2).

High PCV and GCV were recorded for tillers per plant, grains per spike, grain weight per spike, grain yield per plant and harvest index, whereas both PCV and GCV were moderate with respect to spikelets per spike and 1000-grain weight. Grain yield per plant and tillers per plant had high heritability coupled with high genetic advance. High heritability associated with moderate genetic

*Table 1*
*In vitro* screening of doubled haploid lines and their parents for cold tolerance following the TTC test

| Genotypes | Average optical density of formazan | Cold stress response | Genotypes | Average optical density of formazan | Cold stress response |
|---|---|---|---|---|---|
| DH 1 | 0.087 | S | DH 45 | 0.068 | S |
| DH 3 | 0.038 | S | DH 46 | 0.060 | S |
| DH 4 | 0.071 | S | DH 47 | 0.063 | S |
| DH 5 | 0.037 | S | DH 48 | 0.083 | S |
| DH 6 | 0.070 | S | DH 49 | 0.061 | S |
| DH 7 | 0.021 | S | DH 51 | 0.092 | S |
| DH 8 | 0.011 | S | DH 52 | 0.091 | S |
| DH 10 | 0.012 | S | DH 53 | 0.055 | S |
| DH 11 | 0.031 | S | DH 55 | 0.071 | S |
| DH 14 | 0.051 | S | DH 56 | 0.107 | M |
| DH 15 | 0.010 | S | DH 57 | 0.081 | S |
| DH 16 | 0.038 | S | DH 58 | 0.077 | S |
| DH 17 | 0.057 | S | DH 59 | 0.059 | S |
| DH 18 | 0.039 | S | DH 60 | 0.054 | S |
| DH 19 | 0.043 | S | DH 61 | 0.088 | S |
| DH 20 | 0.059 | S | DH 62 | 0.089 | S |
| DH 21 | 0.091 | S | DH 63 | 0.078 | S |
| DH 22 | 0.102 | M | DH 64 | 0.095 | S |
| DH 23 | 0.090 | S | DH 65 | 0.104 | M |
| DH 25 | 0.067 | S | DH 66 | 0.084 | S |
| DH 26 | 0.091 | S | DH 67 | 0.103 | M |
| DH 27 | 0.106 | M | DH 68 | 0.132 | M |
| DH 28 | 0.066 | S | DH 69 | 0.355 | T |
| DH 29 | 0.077 | S | DH 70 | 0.051 | S |
| DH 30 | 0.081 | S | DH 71 | 0.024 | S |
| DH 31 | 0.072 | S | DH 72 | 0.010 | S |
| DH 32 | 0.039 | S | DH 73 | 0.113 | M |
| DH 33 | 0.067 | S | DH 74 | 0.084 | S |
| DH 34 | 0.083 | S | DH 75 | 0.058 | S |
| DH 35 | 0.068 | S | DH 76 | 0.032 | S |
| DH 36 | 0.076 | S | DH 77 | 0.066 | S |
| DH 38 | 0.064 | S | DH 78 | 0.022 | S |
| DH 39 | 0.072 | S | DH 79 | 0.051 | S |
| DH 40 | 0.077 | S | DH 80 | 0.088 | S |
| DH 41 | 0.074 | S | DH 84 | 0.048 | S |
| DH 43 | 0.065 | S | DH 85 | 0.080 | S |
| DH 44 | 0.038 | S | DH 86 | 0.073 | S |
| DH 87 | 0.044 | S | PBW 343 | 0.076 | S |
| DH 88 | 0.047 | S | PW 552 | 0.090 | S |
| DH 89 | 0.073 | S | UP 2418 | 0.044 | S |
| DH 93 | 0.091 | S | Saptdhara | 0.133 | M |
| HPW 42 | 0.053 | S | Sentry | 0.130 | M |
| HPW 89 | 0.061 | S | VWFW 452 | 0.124 | M |
| HPW 147 | 0.067 | S | VWFW 499 | 0.132 | M |
| HPW 155 | 0.068 | S | W 10 | 0.135 | M |
| HPW 184 | 0.038 | S | WW 24 | 0.092 | S |
| HW 3024 | 0.029 | S | | | |

TTC = 2,3,5-triphenyl tetrazolium chloride; S = Susceptible; M = Moderately tolerant; T = Cold-tolerant

*Table 2*
Analysis of variance for different yield-contributing traits of doubled haploids and their parents evaluated for cold tolerance at HAREC, Kukumseri (Lahaul and Spiti)

| Traits | Source | Mean squares | | |
|---|---|---|---|---|
| | Replication | Genotype | Error | |
| | df | 2 | 92 | 184 |
| Tillers/plant (number) | | 0.0162 | 11.0549* | 0.6546 |
| Spikelets/spike (number) | | 12.2950 | 16.7640* | 5.8439 |
| Grains/spike (number) | | 14.1747 | 341.5765* | 13.9384 |
| Grain weight/spike (g) | | 0.0180 | 0.8715* | 0.1690 |
| Grain yield/plant (g) | | 0.1096 | 58.4695* | 0.7006 |
| 1000-grain weight (g) | | 4.7772 | 87.1533* | 2.2984 |
| Harvest index (%) | | 30.2513 | 429.1594* | 11.2667 |

*Significant at $P \leq 0.05$

advance was recorded for grains per spike and harvest index. Grain weight per spike exhibited moderate heritability coupled with moderate genetic advance. Low heritability coupled with low genetic advance for spikelets per spike and high heritability with low genetic advance for 1000-grain weight suggested that these traits are highly influenced by the environment, so selection for these traits may not be effective for yield improvement.

Considering the magnitude of heritability and genetic advance of various yield-contributing traits, selection for tillers per plant, grains per spike, grain yield per plant, harvest index and grain weight per spike may be effective to increase the grain yield (Table 3).

*Correlation and path analysis studies*

Correlation coefficients (Table 4) revealed that grain yield per plant was positively correlated with all the yield-contributing traits, namely tillers per plant, spikelets per spike, grains per spike, grain weight per spike, 1000-grain weight and harvest index. A significant positive association was observed among all the traits, indicating that selection for these yield-contributing traits would result in improved grain yield, as genes tending to enhance these traits would also increase grain yield due to the positive, high genotypic correlation.

*Table 3*
Range, mean and variability parameters for different yield-contributing traits of different genotypes

| Traits | Range | Mean | PCV (%) | GCV (%) | H (%) | GA (%) |
|---|---|---|---|---|---|---|
| Tillers/plant | 2.1–14.0 | 5.5 | 36.65 | 33.62 | 84.1 | 64.00 |
| Spikelets/spike | 8.3–21.3 | 15.6 | 19.70 | 12.21 | 38.4 | 15.58 |
| Grains/spike | 17.2–75.2 | 45.7 | 24.26 | 22.84 | 88.7 | 44.35 |
| Grain weight/spike (g) | 0.629–3.385 | 2.186 | 29.05 | 22.14 | 58.1 | 34.77 |
| Grain yield/plant (g) | 1.880–24.908 | 10.978 | 40.69 | 39.97 | 96.5 | 80.89 |
| 1000-grain weight (g) | 23.77–56.11 | 46.00 | 12.02 | 11.56 | 92.5 | 22.91 |
| Harvest index (%) | 23.50–88.06 | 56.39 | 21.76 | 20.93 | 92.5 | 41.48 |

PCV: Phenotypic coefficient of variation; GCV: Genotypic coefficient of variation; H: Heritability; GA: Genetic advance

*Table 4*
Estimates of direct (diagonal bold) and indirect effects of different yield-contributing traits on grain yield in respect of the genotypes evaluated for cold tolerance

| Traits | Tillers /plant | Spikelets /spike | Grains /spike | Grain weight/spike (g) | 1000-grain weight (g) | Harvest index (%) | Correlation with yield |
|---|---|---|---|---|---|---|---|
| Tillers/plant | **0.555** | 0.002 | 0.350 | –0.291 | 0.100 | 0.080 | 0.796* |
| Spikelets/spike | 0.149 | **0.009** | 1.098 | –1.066 | 0.170 | 0.113 | 0.473* |
| Grains/spike | 0.121 | 0.006 | **1.600** | –1.408 | 0.062 | 0.115 | 0.497* |
| Grain weight/spike (g) | 0.114 | 0.007 | 1.589 | **–1.418** | 0.152 | 0.149 | 0.593* |
| 1000-grain weight (g) | 0.129 | 0.004 | 0.232 | –0.499 | **0.431** | 0.058 | 0.354* |
| Harvest index (%) | 0.137 | 0.003 | 0.566 | –0.652 | 0.077 | **0.324** | 0.455* |

*Significant at $P \leq 0.05$

The further partitioning of these associations into the direct and indirect effects of component characters (Table 4) was done through path coefficient analysis. The positive direct effects of various traits decreased in the order: grains per spike > tillers per plant > 1000-grain weight > harvest index > spikelets per spike. Therefore, selection for these plant traits should be exercised in the aforesaid order to select superior genotypes.

The significant positive correlation of tillers per plant with grain yield was mainly due to its high positive direct effect. However, tillers per plant also showed a positive indirect effect via grains per spike. Spikelets per spike had a very low positive direct effect on grain yield. Its significant positive association with yield was mainly due to its indirect effect via grains per spike, 1000-grain weight, tillers per plant and harvest index. Grains per spike exhibited a very high positive direct effect and a positive indirect effect via tillers per plant and harvest index, resulting in a significant positive correlation with grain yield. The positive correlation of 1000-grain weight and harvest index with grain yield was due to their high positive direct effects as well as to positive indirect effects via all other yield-contributing traits. The positive association of grain weight per spike with grain yield was primarily due to its positive indirect effect via grains per spike, 1000-grain weight, harvest index and tillers per plant. Grain weight per spike had a very high negative direct effect as well as a negative indirect effect on grain yield per plant.

Keeping in view the correlation coefficients of various traits with grain yield in conjunction with path analysis, indirect selection for tillers per plant would be most effective. In addition, harvest index and grains per spike have also been identified as important traits for selection in order to improve grain yield. Selection should not be practised for grain weight per spike as it will adversely affect the grain yield per plant.

*Identification of better genotypes*

All the genotypes performed differently with respect to the various yield-contributing traits. The doubled haploid line DH 36 performed significantly better than both the best parent, VWFW 452, and the check, HPW 155 (released

variety for the region) with respect to tillers per plant, whereas the doubled haploid lines DH 10, DH 41, DH 61, DH 44, DH 45, DH 20, DH 16, DH 23, DH 71, DH 14, DH 39, DH 6, DH 4, DH 8 and DH 47 were at par with the best parent but significantly superior to the check. Various genotypes were identified as performing better for the rest of the parameters (Table 5).

On the basis of the overall ranking of the genotypes with respect to all the traits, only one winter wheat parent, Saptdhara, performed significantly better with respect to all the traits studied, whereas the doubled haploids DH 16, DH 23, DH 20, DH 33, DH 25, DH 6, DH 36, DH 15, DH 63 and DH 10 and the parental genotypes Sentry and WW 24 were identified as performing better for most of the traits (Table 5).

*Table 5*

Top one-third of better performing genotypes with respect to yield-contributing *in vivo* traits evaluated for cold tolerance at HAREC, Kukumseri (Lahaul and Spiti)

| Rank | Tillers/ plant | Spikelets/ spike | Grains/ spike | Grain weight/spike | Grain yield/ plant | 1000-grain weight | Harvest index | Overall basis |
|---|---|---|---|---|---|---|---|---|
| 1 | DH 36*$^{C}$ | DH 22*$^{B}$ | DH 15*$^{C}$ | DH 69*$^{B}$ | DH 38*$^{C}$ | DH 85*$^{C}$ | DH 29*$^{C}$ | Sapt$^{+}$ |
| 2 | DH 10*$^{B}$ | DH 7*$^{B}$ | DH 16*$^{C}$ | DH 33*$^{B}$ | DH 36*$^{C}$ | DH 27*$^{C}$ | DH 39*$^{C}$ | DH 16 |
| 3 | DH 41*$^{B}$ | DH 10*$^{B}$ | DH 11*$^{C}$ | DH 15*$^{B}$ | DH 16*$^{C}$ | DH 84*$^{C}$ | DH 59*$^{C}$ | DH 23 |
| 4 | DH 61*$^{B}$ | Sapt*$^{A+}$ | DH 69*$^{B}$ | DH 16*$^{B}$ | DH 61*$^{C}$ | DH 23*$^{C}$ | DH 33*$^{C}$ | DH 20 |
| 5 | DH 44*$^{B}$ | Sentry* | DH 33*$^{B}$ | Sapt*$^{A+}$ | DH 6*$^{C}$ | DH 25*$^{C}$ | DH 20*$^{C}$ | DH 33 |
| 6 | DH 45*$^{B}$ | DH 11 | DH 35*$^{B}$ | DH 23*$^{B}$ | DH 23*$^{C}$ | DH 36*$^{C}$ | DH 60*$^{B}$ | DH 25 |
| 7 | V452*$^{A+}$ | DH 25 | DH 7*$^{B}$ | Sentry* | DH 20*$^{C}$ | DH 29*$^{C}$ | DH 21*$^{B}$ | DH 6 |
| 8 | DH 20*$^{B}$ | DH 14 | DH 63*$^{B}$ | WW 24* | WW 24*$^{A}$ | DH 5*$^{C}$ | DH 28*$^{B}$ | Sentry |
| 9 | Sapt*$^{+}$ | DH 16 | PW 552*$^{A}$ | DH 86*$^{B}$ | DH 59*$^{B}$ | DH 87*$^{C}$ | Sapt*$^{A+}$ | WW 24 |
| 10 | DH 16*$^{B}$ | DH 34 | DH 10*$^{B}$ | DH 25*$^{B}$ | DH 14*$^{B}$ | DH 56*$^{B}$ | DH 63*$^{B}$ | DH 36 |
| 11 | DH 23*$^{B}$ | DH 87 | DH 32*$^{B}$ | DH 63*$^{B}$ | DH 15*$^{B}$ | DH 20*$^{B}$ | DH 32*$^{B}$ | DH 15 |
| 12 | DH 71*$^{B}$ | DH 15 | DH 72*$^{B}$ | DH 11*$^{B}$ | DH 63*$^{B}$ | DH 80*$^{B}$ | DH 15*$^{B}$ | DH 80 |
| 13 | DH 14*$^{B}$ | DH 30 | DH 23*$^{B}$ | DH 6*$^{B}$ | DH 10* | DH 69*$^{B}$ | W 10* | DH 63 |
| 14 | DH 39*$^{B}$ | DH 3 | DH 87*$^{B}$ | DH 35*$^{B}$ | V452*$^{+}$ | DH 21*$^{B}$ | DH 49*$^{B}$ | DH 61 |
| 15 | DH 6*$^{B}$ | DH 4 | DH 6*$^{B}$ | DH 56 | DH 41* | DH 86*$^{B}$ | DH 80*$^{B}$ | DH 59 |
| 16 | Sentry* | DH 61 | WW 24* | PW 552 | DH 25* | DH 68*$^{B}$ | DH 36* | DH 26 |
| 17 | DH 4*$^{B}$ | DH 26 | DH 86*$^{B}$ | DH 20 | DH 26* | DH 3*$^{B}$ | Sentry* | DH 34 |
| 18 | DH 8*$^{B}$ | DH 6 | DH 18* | DH 32 | Sapt*$^{+}$ | DH 34*$^{B}$ | DH 7* | DH 10 |
| 19 | DH 47*$^{B}$ | DH 21 | DH 25* | DH 87 | DH 80* | DH 4*$^{B}$ | DH 77* | DH 11 |
| 20 | WW 24* | DH 43 | DH 64* | DH 19 | Sentry* | DH 58*$^{B}$ | DH 57* | DH 35 |
| 21 | DH 26 | DH 76 | Sapt*$^{+}$ | DH 29 | DH 4* | Sapt*$^{A+}$ | DH 34* | V452$^{+}$ |
| 22 | DH 49 | DH 33 | DH 19* | DH 10 | DH 33* | DH 65*$^{B}$ | V499*$^{+}$ | DH 65 |
| 23 | DH 57 | DH 78 | Sentry* | DH 65 | DH 28* | DH 61*$^{B}$ | V452*$^{+}$ | DH 18 |
| 24 | DH 79 | DH 1 | DH 65* | DH 59 | DH 35* | DH 22*$^{B}$ | DH 16* | DH 14 |
| 25 | DH 80 | DH 39 | DH 78 | DH 18 | DH 44* | DH 51*$^{B}$ | DH 61 | DH 29 |
| 26 | DH 28 | DH 5 | DH 20 | DH 7 | DH 8* | WW 24* | DH 23 | DH 87 |
| 27 | DH 48 | DH 80 | DH 26 | DH 80 | DH 39* | DH 33*$^{B}$ | DH 22 | DH 4 |
| 28 | DH 63 | DH 64 | DH 56 | DH 58 | DH 18* | DH 28*$^{B}$ | DH 64 | DH 39 |
| 29 | DH 35 | DH 85 | DH 45 | DH 77 | DH 11* | DH 26*$^{B}$ | DH 11 | DH 86 |
| 30 | DH 52 | DH 23 | DH 17 | DH 22 | DH 34* | DH 6*$^{B}$ | DH 65 | W 10 |
| 31 | DH 59 | DH 45 | DH 59 | DH 78 | DH 71* | DH 74 | DH 17 | DH 19 |

*: Significant at P≤0.05, when compared with overall mean; $^{+}$: V452: VWFW 452; Sapt: Saptdhara; V499: VWFW 499; $^{A}$ Best parent; $^{B}$ DH lines at par with the best parent; $^{C}$ DH lines better than the best parent

*Correlation of field performance with* in vitro *parameters*

In order to correlate the field performance of the genotypes with *in vitro* parameters for cold tolerance, the top one-third of better performing genotypes under field conditions (Table 5) were compared with the genotypes screened under *in vitro* conditions. This study revealed that only four parents, namely W 10, Saptdhara, Sentry and VWFW 452, and one doubled haploid line, DH 65, performed better under both the environments. The remaining genotypes behaved differently under *in vitro* and *in vivo* conditions. Since the *in vitro* screening of the doubled haploid lines using the triphenyl tetrazolium chloride (TTC) test, comprising only a single parameter, categorized the genotypes as tolerant or susceptible, the ranking of the genotypes was not possible with respect to this trait. Hence, a rank correlation could not be calculated to correlate the performance of the genotypes under *in vitro* and *in vivo* conditions. Therefore, in addition to the TTC test, other *in vitro* parameters should be identified which clearly distinguish between the cold-tolerant and susceptible genotypes and also correlate well with their performance under field conditions.

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# PROTECTIVE EFFECT OF TWO SYNTHETIC COMPOUNDS AGAINST CHLORSULFURON INJURY IN MAIZE (*Zea mays* L.)

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Two new synthetic compounds – urea (B-3) and thiourea (B-6) derivatives of 4-methylpiperazinyl – were investigated as possible antidotes for chlorsulfuron in maize. It was established that the thiourea derivative was a more effective herbicide protector than the urea derivative. The shoot length of maize plants treated with $10^{-5}$ M chlorsulfuron was inhibited 60% compared to the untreated plants, whereas pretreatment with $5\times10^{-4}$ M B-6 reduced this inhibition to 23%. Moreover, the decrease of 53% in shoot fresh weight caused by herbicide applied alone was countered almost completely by B-6 with only an 11% loss in fresh weight observed. The results of this investigation show that the urea derivative (B-3) appeared to be ineffective in antidoting the injurious effect of chlorsulfuron. Decreases in root length and fresh weight were also offset by B-6. The more favourable protective activity of the thioureido group in B-6 could be due to the formation of an isomer with isothiourea structure, having a sulphydryl group. Treatments with herbicide and B-3 / B-6 alone or in combination had no significant effect on the parameters of the antioxidative defence system tested.

**Key words**: chlorsulfuron, maize, herbicide antidote, antioxidative defence system

## Introduction

Many of the currently available herbicides that are used in practice are not sufficiently selective. To overcome this problem Hoffman (1962) introduced the idea of chemically enhancing the tolerance of the crop (but not the weed) to herbicides, with the use of appropriate herbicide antidotes (Hatzios, 1983).

Chlorsulfuron [1-(2-chlorphenylsulphonyl)-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl) urea] is a sulphonylurea herbicide used to control many broadleaf and certain grass weeds following application at extremely low rates (Hatzios, 1984), but maize (*Zea mays* L.), one of the most important crops in plant production, is sensitive to this herbicide. Because of their high activity and broad weed control spectrum, it would be particularly useful to develop antidotes against sulphonylureas (Devlin and Zbiec, 1990).

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The process of discovering and developing effective herbicide antidotes is quite lengthy. In some cases, the structural similarity of herbicide and safener molecules is apparent (Bordas et al., 2000)

Research on the effect of antidotes on the herbicidal activity of sulphonylureas is limited. In spite of the general interest in herbicide safeners, little information exists on their mode of action. According to Hatzios (1988) safeners may act either as 'bioregulators' influencing the amount of herbicide that reaches its target site in an active form or as 'antagonists' of herbicidal effects at a common site of action (Milhomme and Bastide, 1990).

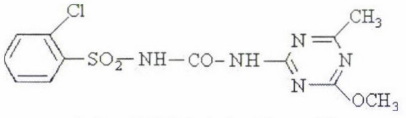
Parker et al. (1980) were the first to observe a safening effect on sulphonylurea herbicides when they showed that naphthalic anhydride could partially protect maize from chlorsulfuron. Chlorsulfuron injury to maize can be alleviated by two oxime antidotes (CGA-43089 and CGA-92194) (Yenne and Hatzios, 1990). Devlin and Zbiec (1990) demonstrated that BAS-145-138 countered the phytotoxic effect of chlorsulfuron in maize. Other data indicated that the herbicide antidote R-25788 provides protection to maize against injury provoked by the herbicide examined in this study (Rubin and Casida, 1985).

The objectives of the research described in this paper were to examine the degree of protection by pretreatments with two synthetic compounds against chlorsulfuron injury in maize and to determine their effect on some parameters of the antioxidant system.

## Materials and methods

*Chemicals*

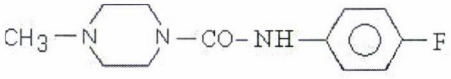
Chlorsulfuron [1-(2-chlorphenylsulphonyl)-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl) urea] has the following chemical formula:



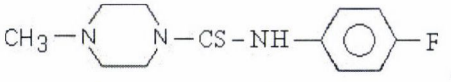
A commercial product containing 75% (w/w) chlorsulfuron was used.

Both the protective compounds used in the present investigation were synthesized and characterized in our laboratory. The purity of the chemicals was greater than 95% as determined by HPLC analysis (Yonova and Stoilkova, 2005).

The urea derivative, B-3 [1-(4-fluorophenylcarbamoyl)-4-methyl-piperazine] had the formula:



while the thiourea derivative, B-6 [1-(4-fluorophenylthiocarbamoyl)-4-methyl-piperazine] had the formula:



All other reagents employed were of analytical grade.

*Plant material and growth conditions*

Maize (*Zea mays* L. cv. Kneja 530) seeds were sterilized and allowed to soak for 24 h in running water. The imbibed seeds were then soaked consecutively in aqueous solutions containing 0.02% (v/v) Tween 80, the potential protecting agents B-3 or B-6 and herbicide for 5 h each. Control seeds were soaked for 10 h in distilled water. The treated seeds were rolled in moist filter paper. The rolls were put into cups with distilled water and placed in darkness at 27°C for two days for germination. Then, the cups with the maize seedlings were moved to a growth chamber (160 $\mu mol\ m^{-2}\ s^{-1}$, 16 h light and 8 h dark, 24 ± 1°C). After 10 days, ten uniform plants were selected for measurements. Their shoot and root lengths and fresh weights were determined.

*Biochemical analyses*

Fresh plant material was immediately extracted and assayed. The hydrogen peroxide content was determined spectrophotometrically by monitoring the absorbance ($A_{390}$) of the KI oxidized product using the method of Alexieva et al. (2001). Lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA), a product of unsaturated fatty acid peroxidation, by the method of Heath and Packer (1968). Chlorophylls and carotenoids were extracted with 80% acetone and estimated according to Arnon (1949). The amounts of free amino acids were determined using the ninhydrine method of Spies (1957).

*Peroxide-scavenging enzyme assays*

Fresh leaf tissue was homogenized in 0.1 M phosphate buffer (pH 7.0) containing 1.0 mM EDTA-Na salt and 1% w/v soluble polyvinylpyrrolidone, and assays were made on the crude extract. Catalase (EC 1.11.1.6) activity was determined by recording the consumption of hydrogen peroxide at 240 nm for 1 min (Beers and Sizer, 1952). Guaiacol peroxidase (EC 1.11.1.7) activity was based on the determination of guaiacol oxidation at 470 nm for 1 min by hydrogen peroxide (Dias and Costa, 1983). Ascorbate peroxidase (EC 1.11.1.11) activity was assayed by measuring the rate of decrease in absorbance at 290 nm for 1 min, i.e. as the rate of ascorbate oxidation by hydrogen peroxide (Nakano and Asada, 1981).

*Statistics*

The data presented are means of three independent experiments with three replications each. The data were analysed statistically and the least significant difference (LSD) was used to evaluate differences between the treatments.

## Results and discussion

### *Effect of B-6 and B-3 in countering chlorsulfuron inhibition in maize*

At the whole-plant level, the effect of chlorsulfuron stress is usually perceived as a decrease in growth. Growth analysis allows a preliminary evaluation of the degree of damage induced by stress effects in the main physiological processes of stressed plants. The dose-response curve for five different concentrations of chlorsulfuron (Fig. 1) shows that a concentration of $10^{-5}$ M caused a 50% inhibition in the shoot growth of maize plants, so chlorsulfuron was applied at a concentration of $10^{-5}$ M in all the experiments.

Chlorsulfuron applied alone caused a significant inhibition (50% of the control) in the linear shoot growth of the maize plants. Maize plants treated with B-6 at $10^{-3}$ and $10^{-4}$ M had 10 and 14% greater shoot length, respectively, while the $5\times10^{-4}$ M concentration caused a slight reduction in shoot length (5%) compared with the untreated plants. Pretreatment of seeds with all three concentrations of B-6 ($10^{-3}$, $5\times10^{-4}$ and $10^{-4}$ M) reduced the growth inhibition caused by herbicide to 20, 23 and 41%, respectively.

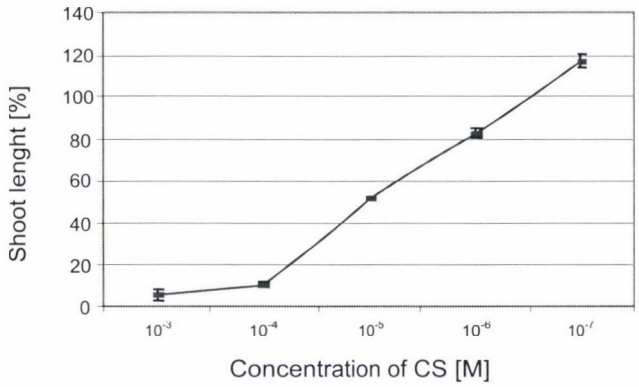



*Fig. 1.* Dose-response curve for application of chlorsulfuron (CS) after seed treatment in maize

The safening effect of B-6 was also observed when the shoot fresh weight was measured (Table 1). The 53 % decrease in shoot fresh weight caused by the herbicide was countered almost completely by $10^{-3}$ and $5\times10^{-4}$ M of B-6, with only 8 and 11% losses in fresh weight observed.

Shoot growth is more susceptible to chlorsulfuron than root growth. The tested herbicide caused a 48% reduction in the length of the root system and a 46% decrease in fresh weight (Table 1). The decreases in root length and fresh weight were offset by all three concentrations of B-6.

The results of the investigation revealed an antagonistic interaction between B-6 and the herbicide.

Although the most active antidote concentration was $10^{-3}$ M, a concentration of $5\times10^{-4}$ M was chosen for all subsequent studies.

*Table 1*

Safening influence of 1-(4-fluorophenylthiocarbamoyl)-4-methylpiperazine (B-6) on the herbicidal effect of chlorsulfuron (CS) on the growth of maize (Means ± SE, n=6)

| CS (M) | B-6 (M) | Shoots | | | | Roots | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Length | | F W | | Length | | F W | |
| | | (mm) | (%) | (mg plant$^{-1}$) | (%) | (mm) | (%) | (mg plant$^{-1}$) | (%) |
| 0 | 0 | 155 ± 1.206 | 100 | 390 ± 2.123 | 100 | 99 ± 0.012 | 100 | 96 ± 0.012 | 100 |
| $10^{-5}$ | 0 | 62 ± 0.204 | 40 | 185 ± 1.569 | 47 | 51 ± 0.103 | 52 | 54 ± 0.065 | 56 |
| 0 | $10^{-3}$ | 170 ± 1.230 | 110 | 455 ± 0.145 | 117 | 71 ± 0.098 | 72 | 135 ± 0.325 | 141 |
| 0 | $5\times10^{-4}$ | 148 ± 0.026 | 95 | 436 ± 0.698 | 112 | 99 ± 0.012 | 100 | 153 ± 0.254 | 159 |
| 0 | $10^{-4}$ | 177 ± 1.236 | 114 | 433 ± 0.684 | 234 | 127 ± 0.96 | 128 | 167 ± 0.231 | 174 |
| $10^{-5}$ | $10^{-3}$ | 124 ± 0.561 | 80 | 358 ± 0.781 | 92 | 90 ± 0.147 | 91 | 123 ± 0.562 | 128 |
| $10^{-5}$ | $5\times10^{-4}$ | 119 ± 1.145 | 77 | 349 ± 1.001 | 89 | 87 ± 0.451 | 88 | 139 ± 0.214 | 145 |
| $10^{-5}$ | $10^{-4}$ | 92 ± 0.457 | 59 | 251 ± 1.000 | 64 | 60 ± 0.562 | 61 | 68 ± 0.254 | 71 |
| $LSD_{5\%}$ | | 6.436 | | 2.30 | | 5.621 | | 3.125 | |
| $LSD_{1\%}$ | | 8.547 | | 3.317 | | 7.465 | | 4.337 | |

Different inter-treatment times (IT) between the B-6 and herbicide treatments were also tested (Table 2). Herbicide treatment was carried out 0, 12 and 24 h after B-6 treatment. The shoot and root lengths of maize plants treated with chlorsulfuron after 24 h were inhibited by 48 and 41%, respectively. These plants also showed a reduction in shoot and root fresh weights. Partial protection of the maize plants was observed when the herbicide treatment was performed after 12 h, while the greatest protective effect of B-6 was found at IT = 0 h, i.e. herbicide treatment of the seeds immediately followed the safener treatment. It is thus clear that a longer period should not clapse between the application of the two compounds (safener and herbicide).

The urea analogue of B-6, B-3, was also assayed as a possible antidote to chlorsulfuron. Both compounds have the same structural elements but the nature of the bridge between the phenyl and piperazine rings is different. Shoot length was inhibited 50% by chlorsulfuron and 68% by the combination of herbicide and B-3 compared with the untreated plants. The same trend was evident in respect to the fresh weight, the decrease being 36% for herbicide treatment and 60% for the combination. The inhibition of maize root elongation and fresh weight by chlorsulfuron, 52 and 38%, respectively, compared to the control plants, was not reduced when the seeds were pretreated with B-3 (Table 3). These results show that the urea analogue B-3 had no antidote activity against chlorsulfuron under the conditions of this test. Thus, the structural similarity (urea bridge) of this safener to chlorsulfuron did not appear to support the "competitive antagonism" theory proposed to explain the safening action (Hatzios, 1984).

*Table 2*

Safening influence of 1-(4-fluorophenylthiocarbamoyl)-4-methylpiperazine (B-6) on the herbicidal effect of chlorsulfuron (CS) on the growth of maize depending on the time between the application of herbicide and the tested compound (Means ± SE, n=6)

| Time* | CS (M) | B-6 (M) | Shoots | | | | Roots | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Length | | F W | | Length | | F W | |
| | | | (mm) | (%) | (mg plant$^{-1}$) | (%) | (mm) | (%) | (mg plant$^{-1}$) | (%) |
| – | 0 | 0 | 133 ± 1.102 | 100 | 355 ± 2.365 | 100 | 80 ± 0.235 | 100 | 102 ± 0.961 | 100 |
| – | $10^{-5}$ | 0 | 67 ± 0.965 | 50 | 206 ± 1.965 | 58 | 51 ± 0.123 | 64 | 65 ± 0.213 | 64 |
| – | 0 | $5\times10^{-4}$ | 138 ± 0.745 | 104 | 395 ± 1.236 | 111 | 89 ± 1.421 | 111 | 145 ± 0.452 | 142 |
| 0 | $10^{-5}$ | $5\times10^{-4}$ | 108 ± 0.874 | 81 | 318 ± 0.998 | 90 | 69 ± 0.065 | 86 | 97 ± 0.211 | 95 |
| 12 | $10^{-5}$ | $5\times10^{-4}$ | 75 ± 0.236 | 56 | 227 ± 1.102 | 64 | 50 ± 0.214 | 63 | 57 ± 0.0654 | 56 |
| 24 | $10^{-5}$ | $5\times10^{-4}$ | 56 ± 0.345 | 42 | 169 ± 0.896 | 48 | 47 ± 0.036 | 59 | 48 ± 0.0231 | 47 |
| | $LSD_{5\%}$ | | 7.93 | | 5.012 | | 1.579 | | 1.571 | |
| | $LSD_{1\%}$ | | 10.412 | | 6.623 | | 2.246 | | 2.234 | |

*between application of B-6 and CS (h)

*Table 3*

Safening influence of 1-(4-fluorophenylcarbamoyl)-4-methylpiperazine (B-3) on the herbicidal effect of chlorsulfuron (CS) on the growth of maize (Means ± SE, n=6)

| CS (M) | B-3 (M) | Shoots | | | | Roots | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Length | | F W | | Length | | F W | |
| | | (mm) | (%) | (mg plant$^{-1}$) | (%) | (mm) | (%) | (mg plant$^{-1}$) | (%) |
| 0 | 0 | 180 ± 0.691 | 100 | 429 ± 1.236 | 100 | 186 ± 0.236 | 100 | 129 ± 0.962 | 100 |
| $10^{-5}$ | 0 | 90 ± 0.123 | 50 | 273 ± 1.141 | 64 | 90 ± 0.114 | 48 | 80 ± 0.564 | 62 |
| 0 | $5\times10^{-4}$ | 124 ± 0.692 | 69 | 326 ± 1.952 | 76 | 124 ± 0.865 | 67 | 114 ± 0.486 | 88 |
| $10^{-5}$ | $5\times10^{-4}$ | 57 ± 0.235 | 32 | 171 ± 0.987 | 40 | 51 ± 0.036 | 27 | 87 ± 1.006 | 67 |
| $LSD_{5\%}$ | | 8.586 | | 5.691 | | 10.868 | | 1.913 | |
| $LSD_{1\%}$ | | 11.434 | | 8.621 | | 14.488 | | 2.898 | |

Studies on the mode of action of chlorsulfuron have shown that this herbicide appears to block the activity of acetolactate synthase (ALS, EC 4.1.3.18), leading to a decrease in the levels of three amino acids (valine, leucine and isoleucine). Consequently, the protein synthesis so necessary for plants is eventually terminated and the treated plant dies (Devlin and Zbiec, 1991). However, Clayton and Reynolds (1991) reported that protein synthesis is not inhibited by chlorsulfuron. According to Royuela et al. (1991) chlorsulfuron creates a shortage of branched amino acids that could, in turn, activate protein catabolism, so that there is an increase in the free amino acid pool.

The present data showed that the content of free amino acids in chlorsulfuron-treated plants was 63% higher than in the control (Table 4). These results basically agree with those of a number of investigators (Rhodes et al., 1987; Royuela et al., 1991; Scarponi et al., 1997). These authors hypothesized that the increases in free amino acid levels in samples treated with herbicide were due to the degradation of pre-existing proteins, rather than to the synthesis of new amino acids.

*Table 4*

Effects of chlorsulfuron (CS) and B-3 / B-6 (Compd) alone and in combination on the levels of free amino acids, carotenoids and chlorophyll (*a*+*b*) in maize leaves (Means ± SE, n=6)

| CS (M) | Compd $5\times10^{-4}$ M | Free amino acids (μmol leucine eq g$^{-1}$ FW) | | Carotenoids (mg g$^{-1}$ FW) | | Chlorophyll (*a*+*b*) (mg g$^{-1}$ FW) | |
|---|---|---|---|---|---|---|---|
| | | (mg g$^{-1}$ FW) | (%) | (μmol g$^{-1}$ FW) | (%) | (μmol g$^{-1}$ FW) | (%) |
| 0 | 0 | 20.397 ± 2.123 | 100 | 0.229 ± 0.004 | 100 | 2.554 ± 0.069 | 100 |
| $10^{-5}$ | 0 | 33.157 ± 3.545 | 163 | 0.209 ± 0.006 | 91 | 1.706 ± 0.107 | 67 |
| 0 | B-3 | 5.501 ± 0.129 | 27 | 0.216 ± 0.003 | 94 | 3.998 ± 0.105 | 117 |
| $10^{-5}$ | B-3 | 12.595 ± 4.564 | 62 | 0.211 ± 0.042 | 92 | 3.049 ± 0.099 | 119 |
| 0 | B-6 | 28.166 ± 2.105 | 138 | 0.316 ± 0.01 | 138 | 4.110 ± 0.068 | 161 |
| $10^{-5}$ | B-6 | 28.156 ± 3.140 | 138 | 0.316 ± 0.009 | 138 | 3.770 ± 0.101 | 148 |
| $LSD_{5\%}$ | | 1.036 | | 0.0161 | | 0.195 | |
| $LSD_{1\%}$ | | 1.409 | | 0.0217 | | 0.262 | |

When applied alone or in combination with herbicide, B-3 treatment led to decreases of 73% and 38%, respectively, in the level of free amino acids compared with the control. By contrast, B-6 induced the accumulation of free amino acids (38% over the control), but to a lesser extent than the herbicide. The amount of free amino acids observed in the case of the combined application of B-6 and herbicide was equal to that recorded when the safener was applied alone.

*Effect of B-6, B-3 and chlorsulfuron on parameters of the antioxidant system in maize*

Under certain abiotic stress conditions, including ozone, herbicides, high light, plant pathogens and heavy metals (Mock et al., 1998), the level of active oxygen species (AOS) may increase, resulting in oxidative damage to membrane lipids, DNA and proteins. There must be a balance between the production of AOS and their removal by the antioxidant system. This system includes enzymatic and non enzymatic compounds (Asada, 1996).

Changes were examined in the content of oxidative parameters (MDA, $H_2O_2$) and in the activity of antioxidant enzymes (CAT, APX, GPX) in maize plants treated with herbicide and safeners, separately and in combination.

The results showed that chlorsulfuron reduced the chlorophyll (*a+b*) content (33%), whereas B-3 and B-6 increased it (by 17 and 61%, respectively) (Table 4). Combined treatment with herbicide and B-3/B-6 increased the chlorophyll (a+b) content by 19%/48% over the control. Plants treated with B-6 (alone or combined with herbicide) were characterized by a higher content of carotenoids (38% over the control) (Table 4).

In chlorsulfuron-treated plants the activities of the peroxidases (APX and GPX) were similar to or slightly lower than those in the control plants (Table 5), while this form of stress had an insignificant effect on the specific CAT activity (5% increase). These results demonstrated that the APX, GPX and CAT activities were not sensitive to chlorsulfuron injury.

*Table 5*

Changes in the specific activities of $H_2O_2$-scavenging enzymes (CAT, APX and GPX) in the leaves of maize after seed treatment with chlorsulfuron (CS) and B-3/B-6 (Compd) alone and in combination (Means ± SE, n=6)

| CS (M) | Compd $5\times10^{-4}$ M | CAT | | APX | | GPX | |
|---|---|---|---|---|---|---|---|
| | | µmol decomp $H_2O_2$ $mg^{-1}$ protein $min^{-1}$ | (%) | µmol oxid guaiacol $mg^{-1}$ protein $min^{-1}$ | (%) | µmol oxid ascorbate $mg^{-1}$ protein $min^{-1}$ | (%) |
| 0 | 0 | 25.065 ± 0.883 | 100 | 2.068 ± 0.104 | 100 | 0.835 ± 0.06 | 100 |
| $10^{-5}$ | 0 | 26.203 ± 0.565 | 105 | 1.841 ± 0.111 | 89 | 0.765 ± 0.062 | 92 |
| 0 | B-3 | 28.218 ± 1.000 | 112 | 1.361 ± 0.202 | 66 | 0.693 ± 0.075 | 83 |
| $10^{-5}$ | B-3 | 19.605 ± 0.471 | 78 | 2.2.275 ± 0.118 | 110 | 1.222 ± 0.07 | 146 |
| 0 | B-6 | 18.229 ± 0.854 | 73 | 0.865 ± 0.041 | 42 | 0.607 ± 0.096 | 73 |
| $10^{-5}$ | B-6 | 49.508 ± 0.196 | 198 | 1.077 ± 0.067 | 52 | 0.780 ± 0.072 | 93 |
| $LSD_{5\%}$ | | 1.562 | | 1.800 | | 0.280 | |
| $LSD_{1\%}$ | | 2.103 | | 2.423 | | 0.377 | |

Treatment with B-3 alone decreased the specific APX and GPX activities by 34 and 17% compared to the untreated plants (Table 5). The activities of both enzymes were higher in maize treated with a combination of B-3 and herbicide (10% and 46%, respectively) than in the control plants. Probably this stimulation of both peroxidases led to the decrease in the $H_2O_2$ content (to 53% of the control) in these plants (Fig. 2). The fact that the activities of these $H_2O_2$-scavenging enzymes were higher in maize treated with B-3+chlorsulfuron suggests that the cells of these plants release hydrogen peroxide more rapidly than the control cells.

Treatment of maize seeds with B-6 caused a decrease in the activity of all the antioxidants by 27% for CAT and GPX and by 58% for APX compared to the control maize plants (Table 5). These results were accompanied by a slight (6%) increase of the $H_2O_2$ content in plants treated with B-6. Despite the higher activities of CAT (98% over the control) in plants treated with a combination of herbicide and B-6, the level of $H_2O_2$ remained at 27% over the control (Fig. 2). This correlated well with the low activities of APX and GPX in these plants. Prasad et al. (1994) suggested that the accumulation of peroxide signals the production of antioxidant enzymes such as CAT. In plants treated with B-6 there were already symptoms of moderate oxidative stress. The accumulation of $H_2O_2$ occurred first and then, probably as a response, CAT activity increased. Probably, this level of hydrogen peroxide is able to function exclusively as a secondary messenger, and is not yet cytotoxic.

A high $H_2O_2$ concentration combined with other AOS could increase lipid peroxidation (Leshem, 1984; Markhart, 1986; Asada and Takahashi, 1987). The present results (Fig. 2) basically agree with those reported by these authors, showing an increase of 27% over the untreated control in the MDA levels of variants treated with B-6 and herbicide. The MDA content remained almost unchanged in plants pretreated with B-3.

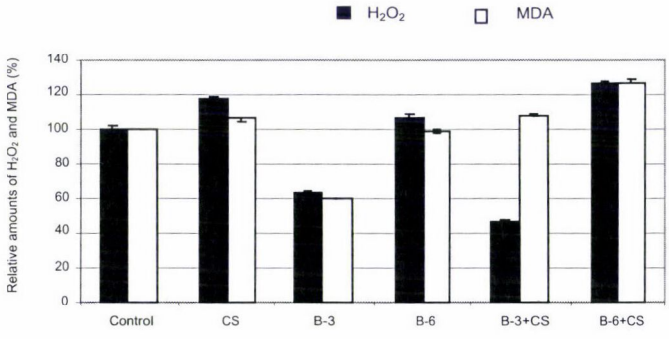



*Fig. 2.* Influence of chlorsulfuron (CS) and synthetic compounds (B-3 /B-6) applied alone and in combination on the levels of $H_2O_2$ and MDA in the leaves of maize. Control: $H_2O_2$ = 567.802 nmol $g^{-1}$ FW ($LSD_{5\%}$ = 1.275; $LSD_{1\%}$ = 1.723); MDA = 55.61 nmol $g^{-1}$ FW ($LSD_{5\%}$ = 0.708; $LSD_{1\%}$ = 0.958)

In conclusion, the results presented in this paper have demonstrated for the first time the antidote activity of two new synthetic compounds – the urea (B-3) and thiourea (B-6) derivatives of 4-methylpiperazinyl against chlorsulfuron in maize. It was established that the thiourea derivate was a highly effective antidote to this herbicide, apparently due to the thiourea bridge, which is more favourable for protective activity. The urea derivate appeared to be ineffective in countering the injurious effect of chlorsulfuron in maize. The mechanism through which B-6 protects maize against chlorsulfuron injury is not yet understood, but it seems probable that it does not involve the activation of the antioxidant defence system.

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# PROPERTIES OF HUMIC AND FULVIC ACIDS UNDER THE LONG-TERM USE OF FERTILIZERS IN A RICE-WHEAT-COWPEA SYSTEM ON A MOLLISOL

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The properties of humic (HA) and fulvic acids (FA) isolated from a 27-year-old long-term experiment with rice-wheat-cowpea on a Mollisol in northern India were evaluated for elemental composition and functional groups. As compared to fallow, C, H and N decreased, while O increased in the control, but the use of NPK+FYM over the years enhanced the C and N of humic substances. The carboxylic (-COOH) and phenolic-OH groups declined in the control, but improved with NPK+FYM in comparison to fallow. Carboxylic groups (-COOH) contributed about 2–3 times more in HA and 4–5 times more in FA than the respective phenolic-OH groups to the molecular weights of these fractions of soil organic matter. The results suggested the role of integrated nutrient management in maintaining active soil humus over the years.

**Key words:** humic acid, fulvic acid, long-term fertilizer use, rice-wheat-cowpea

## Introduction

Soil organic matter (SOM) acts as a nutrient supplier, soil conditioner, environmental protector, substrate for microorganisms, and determinant of sustainable productivity (Schnitzer, 1991). Continuous cultivation for 35 years reduced the synthesis of humic substances, but enhanced the clay-bound organic matter in the soil (Skjemstad et al., 1986). Regular manuring for 22 years increased humification (Govi et al., 1992). The humus of manured plots in eight long-term experiments located at different sites was more oxidized than that of the fertilizer plots (Gonet and Wegner, 1993). In a 24-year-old experiment, the organic matter improved under well-fertilized crop rotations due to better root growth and the return of more plant residues to the soil, but it remained constant under fallow and continuous wheat (Campbell and Zentner, 1993).

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As information on the quality of soil humus is very important, the present study was conducted to monitor the properties of the humic (HA) and fulvic acid (FA) fractions of SOM for elemental composition and functional groups in a long-term experiment established in 1971 with rice-wheat-cowpea cropping on a Mollisol.

## Materials and methods

Surface and subsurface soil samples at 0–15 and 15–30 cm depths were collected from a 27-year-old long-term fertilizer experiment with a rice-wheat-cowpea system. This permanent field experiment is situated in North India at Pantnagar in the foothills of the Himalayas (29°N, 79.3°E, 284 masl) with a humid subtropical climate on an Aquic Hapludoll soil. Initially, the experimental soil had pH 7.3, EC 0.35 dS $m^{-1}$, organic carbon 1.48%, CEC 20 cMole ($p^+$) $kg^{-1}$ and silty clay loam texture (Nand Ram, 1995).

The following treatments, quadruplicated in a randomized block design, were selected for this study:

| | |
|---|---|
| Fallow | No crop, no fertilizers or manure |
| Control | Cropping without fertilizers or manure |
| NPK | Cropping with optimal N, P and K fertilizers |
| NPK+FYM | Cropping with optimal N, P and K fertilizers along with farmyard manure (FYM) |

Based on initial soil tests, N, P and K were added only to rice and wheat @ 120, 26 and 33 kg $ha^{-1}$, respectively, in both crops separately in the form of urea, single superphosphate and muriate of potash. Cowpea was grown as a fodder crop on residual soil fertility. Farmyard manure (FYM) was applied @ 15 t $ha^{-1}$ $year^{-1}$ before sowing wheat.

Humic substances were extracted from soil samples after initial decalcification by repeated treatment with 0.01 *N* NaOH, and fractionated into HA and FA by acidifying the alkaline extract. Humic acid was purified by redissolving in 0.01 *N* NaOH, followed by centrifugation, treatment with HF+HCl, dialysis against distilled water and finally drying. Fulvic acid was purified by adsorption on activated charcoal, washing with 1 *N* $H_2SO_4$ until free of $Fe^{2+}$, eluting with 1 *N* $NH_4OH$ and finally drying (Nand Ram and Raman, 1984).

In both HA and FA, C, H and N were determined using a CHN analyser and O was computed as the remainder. Total acidity and carboxylic (-COOH) groups were measured by the $Ba(OH)_2$ and $Ca(OAc)_2$ methods, respectively, while phenolic-OH groups were obtained by subtracting the carboxyls from the total acidity (Schnitzer and Gupta, 1965). Elemental composition and the analysis of functional groups were utilized to derive the molecular formula of HA and FA following the procedure of Schnitzer and Desjardins (1962), and using the respective molecular weights of 1000 and 670 (Nand Ram and Raman, 1981).

## Results and discussion

### *Elemental composition*

Data on the elemental composition of HA and FA as influenced by the continuous use of fertilizers and manures in rice and wheat for 27 years are presented in Table 1. In HA the values of C, H, N and O ranged from 51.00–55.60, 4.00–5.40, 3.50–4.70 and 34.30–41.50% at 0–15 cm and from 49.50–54.00, 5.20–6.10, 3.10–4.10 and 35.50–42.20% at 15–30 cm soil depths, respectively, while in FA, the percentage of the respective elements varied from 39.00–44.40, 3.80–5.00, 2.80–4.30 and 46.30–54.40 at 0–15 cm, and from 42.20–46.60, 4.50–5.50, 3.00–4.40 and 43.50–50.30 at 15–30 cm depths.

*Table 1*
Elemental composition (%) of humic and fulvic acids

| Treatment | Soil depth (cm) | C | H | N | O |
|---|---|---|---|---|---|
| | | | Humic acid | | |
| Fallow | 0–15 | 52.10 | 5.00 | 4.00 | 38.90 |
| | 15–30 | 50.30 | 5.60 | 3.70 | 40.40 |
| Control | 0–15 | 51.00 | 4.00 | 3.50 | 41.50 |
| | 15–30 | 49.50 | 5.20 | 3.10 | 42.20 |
| NPK | 0–15 | 52.50 | 4.60 | 4.40 | 38.50 |
| | 15–30 | 50.50 | 5.50 | 4.00 | 40.00 |
| NPK+FYM | 0–15 | 55.60 | 5.40 | 4.70 | 34.30 |
| | 15–30 | 54.00 | 6.10 | 4.40 | 35.50 |
| $CD_{5\%}$ | 0–15 | 0.95 | 0.25 | 0.62 | 1.20 |
| | 15–30 | 0.54 | 0.12 | 0.37 | 0.59 |
| | | | Fulvic acid | | |
| Fallow | 0–15 | 41.40 | 4.50 | 3.20 | 50.90 |
| | 15–30 | 42.70 | 4.90 | 3.10 | 49.30 |
| Control | 0–15 | 39.00 | 3.80 | 2.80 | 54.40 |
| | 15–30 | 42.20 | 4.50 | 3.00 | 50.30 |
| NPK | 0–15 | 43.40 | 4.50 | 3.90 | 48.20 |
| | 15–30 | 45.10 | 5.00 | 4.00 | 45.90 |
| NPK+FYM | 0–15 | 44.40 | 5.00 | 4.30 | 46.30 |
| | 15–30 | 46.60 | 5.50 | 4.40 | 43.50 |
| $CD_{5\%}$ | 0–15 | 0.31 | 0.40 | 0.41 | 5.83 |
| | 15–30 | 0.73 | 0.34 | 0.53 | 4.09 |

The results of elemental analysis revealed C and O to be the major constituents of both HA and FA. In addition, more C and less O was recorded in HA than in the corresponding FA extracted from the same treatments at both soil depths. This is because HA, being a first condensation product, contains more methoxy groups, whereas the synthesis of FA is accomplished by the loss of C and a gain in O (Rautela and Nand Ram, 2000). Relatively, the NPK+FYM treatment showed a significantly higher percentage of C in HA and FA at both soil depths, suggesting the humus of this treatment to be more mature as compared to the fallow, fertilized and unfertilized treatments. Such variations may be ascribed to the differences in their synthesis under various treatments (Das and Nand Ram, 2006).

In HA and FA isolated from the control treatment, where neither fertilizers nor manures have been added since the inception of this permanent field experiment, a marked decline in C, H and N, but an improvement in O was observed as compared to the fallow treatment. This might be due to the loss of C and a gain in O both by oxidation and transformation as a result of intensive cultivation (Kononova, 1966).

The joint use of fertilizers and farmyard manure (NPK+FYM) led to a considerable increase in C and N but to a decrease in O in both HA and FA in comparison to the fallow treatment. The addition of fertilizers alone (NPK) also improved N in both HA and FA. Such variations may be due either to the incomplete hydrolysis of proteinaceous materials or to the involvement of heterocyclic nitrogenous constituents.

*Functional groups*

Oxygen-containing functional groups, i.e. total acidity, carboxylic and phenolic-OH groups, were comparatively more abundant in FA than in HA in the same treatment at both the soil depths (Table 2). The increase in total acidity with decreasing molecular weight is consistent with the enhanced oxidation of the low molecular weight fractions of SOM (Chen et al., 1977).

The content of carboxylic (-COOH) groups was higher (4.00–5.20 and 4.70–5.60 me $g^{-1}$) in FA as compared with 2.60–3.90 and 3.40–4.00 me $g^{-1}$ in HA at 0–15 and 15–30 cm depths, respectively. It is also evident that the carboxyl groups were significantly more abundant in FAs as compared to the corresponding HAs. This could be attributed to the low particle weight of FA, as decarboxylation did not occur before polymerization to HA. The oxidative disintegration of HA may possibly also lead to the higher content of carboxyl groups in FA (Flaig et al., 1975).

On comparing the functional groups of the control treatment with those of the fallow treatment, reductions of 0.40 and 0.20 me $g^{-1}$ for the carboxylic groups and 0.40 and 0.10 me $g^{-1}$ for the phenolic-OH groups was noted at the 0–15 and 15–30 cm depths, respectively, in HA. The corresponding decline in FA was 0.40 and 0.30 me $g^{-1}$ for the carboxylic groups and 0.20 and 0.40 me $g^{-1}$ for phenolic-OH groups at the respective soil depths, while the integrated use of fertilizers and manures (NPK+FYM) regularly for 27 years led to an increase of 0.90 and 0.40 me $g^{-1}$ in HA and 0.80 and 0.60 me $g^{-1}$ in FA for the carboxylic groups at 0–15 and 15–30 cm soil depths, respectively. For the phenolic-OH groups, the improvement was of the order of 0.10 me $g^{-1}$ in HA and 0.20 me $g^{-1}$ in FA for each soil depth. Gonet and Wegner (1993) also reported that the humic substances in manured soils remained in a higher oxidation state than in soils under NPK fertilizers.

*Molecular formula*

The molecular formulas of HA and FA (Table 3) were computed from their elemental and functional group analysis. These formulas express the relationships of the functional groups to the unreactive nuclear matrix of the macromolecules. It is interesting to note that a relatively higher percentage (25.17–32.17) of the molecular weight was made up by functional groups in FA than in HA (18.20–24.33). Moreover, the carboxylic groups (-COOH) contributed about 2–3 times more to the molecular weights of these fractions of SOM in HA and 4–5 times more in FA than the respective phenolic-OH groups. The contribution of functional groups to the molecular weight of humic substances under different treatments was found to be significantly higher under NPK+FYM, particularly for the carboxylic groups at both soil depths, indicating the humic substances to be largely aliphatic, more hydrophilic and potentially more active as compared to other treatments.

*Table 2*
Oxygen-containing functional groups (me $g^{-1}$) in humic and fulvic acids

| Treatment | Soil depth (cm) | Total acidity | -COOH groups | Phenolic-OH groups |
|---|---|---|---|---|
| | | | Humic acid | |
| Fallow | 0–15 | 6.10 | 3.00 | 3.10 |
| | 15–30 | 6.50 | 3.60 | 2.90 |
| Control | 0–15 | 5.30 | 2.60 | 2.70 |
| | 15–30 | 6.20 | 3.40 | 2.80 |
| NPK | 0–15 | 5.70 | 2.70 | 3.00 |
| | 15–30 | 6.40 | 3.50 | 2.90 |
| NPK+FYM | 0–15 | 7.10 | 3.90 | 3.20 |
| | 15–30 | 7.00 | 4.00 | 3.00 |
| $CD_{5\%}$ | 0–15 | 0.09 | 0.19 | 0.21 |
| | 15–30 | 0.27 | 0.24 | 0.13 |
| | | | Fulvic acid | |
| Fallow | 0–15 | 7.60 | 4.40 | 3.20 |
| | 15–30 | 8.10 | 5.00 | 3.10 |
| Control | 0–15 | 7.00 | 4.00 | 3.00 |
| | 15–30 | 7.40 | 4.70 | 2.70 |
| NPK | 0–15 | 7.70 | 4.60 | 3.10 |
| | 15–30 | 7.80 | 4.80 | 3.00 |
| NPK+FYM | 0–15 | 8.60 | 5.20 | 3.40 |
| | 15–30 | 8.90 | 5.60 | 3.30 |
| $CD_{5\%}$ | 0–15 | 0.10 | 0.18 | 0.25 |
| | 15–30 | 0.07 | 0.08 | 0.07 |

*Table 3*
Molecular formulae and percentage contribution of functional groups to the molecular weight (MW) of humic and fulvic acids

| Treatment | Soil depth (cm) | Molecular formulae | MW | Percentage contribution to MW from | | |
|---|---|---|---|---|---|---|
| | | | | Functional groups | | Nuclear matrix |
| | | | | -COOH | Phenolic-OH | |
| | | | Humic acid | | | |
| Fallow | 0–15 | $C_{40}H_{44}N_3O_{15}(COOH)_3(OH)_3$ | 992 | 13.60 | 5.14 | 81.26 |
| | 15–30 | $C_{38}H_{49}N_3O_{11}(COOH)_4(OH)_3$ | 954 | 13.86 | 5.34 | 75.80 |
| Control | 0–15 | $C_{40}H_{34}N_3O_{17}(COOH)_3(OH)_3$ | 1014 | 13.31 | 5.02 | 81.67 |
| | 15–30 | $C_{38}H_{46}N_2O_{17}(COOH)_3(OH)_3$ | 988 | 13.66 | 5.16 | 81.18 |
| NPK | 0–15 | $C_{41}H_{40}N_3O_{15}(COOH)_3(OH)_3$ | 1000 | 13.10 | 5.10 | 81.80 |
| | 15–30 | $C_{38}H_{48}N_3O_{14}(COOH)_4(OH)_3$ | 1001 | 13.98 | 5.09 | 76.93 |
| NPK+FYM | 0–15 | $C_{42}H_{47}N_3O_{10}(COOH)_4(OH)_3$ | 984 | 18.29 | 5.18 | 76.53 |
| | 15–30 | $C_{43}H_{53}N_3O_{10}(COOH)_4(OH)_4$ | 1019 | 17.66 | 6.67 | 75.67 |
| $CD_{5\%}$ | 0–15 | | 3.84 | 0.05 | 0.02 | 0.92 |
| | 15–30 | | 3.54 | 0.07 | 0.02 | 0.88 |
| | | | Fulvic acid | | | |
| Fallow | 0–15 | $C_{20}H_{25}NO_{13}(COOH)_3(OH)_2$ | 656 | 20.57 | 5.18 | 74.25 |
| | 15–30 | $C_{21}H_{28}NO_{13}(COOH)_3(OH)_2$ | 671 | 20.11 | 5.06 | 74.83 |
| Control | 0–15 | $C_{19}H_{20}NO_{15}(COOH)_3(OH)_2$ | 671 | 20.11 | 5.06 | 74.83 |
| | 15–30 | $C_{21}H_{25}NO_{13}(COOH)_3(OH)_2$ | 668 | 20.20 | 5.08 | 74.72 |
| NPK | 0–15 | $C_{21}H_{25}N_2O_{12}(COOH)_3(OH)_2$ | 666 | 20.27 | 5.10 | 74.63 |
| | 15–30 | $C_{22}H_{29}N_2O_{11}(COOH)_3(OH)_2$ | 666 | 20.27 | 5.10 | 74.63 |
| NPK+FYM | 0–15 | $C_{22}H_{28}N_2O_{11}(COOH)_3(OH)_2$ | 665 | 20.30 | 5.11 | 74.59 |
| | 15–30 | $C_{22}H_{31}N_2O_8(COOH)_4(OH)_2$ | 665 | 27.06 | 5.11 | 67.83 |
| $CD_{5\%}$ | 0–15 | | 4.22 | 0.13 | 0.05 | 0.78 |
| | 15–30 | | 2.66 | 0.07 | 0.03 | 0.72 |

## Conclusions

Continuous cropping of rice-wheat-cowpea for 27 years without any nutrient input reduced C, H and N but improved O in the HA and FA of the control plot owing to a loss in C and a gain in O due to intensive cultivation over the years. However, the joint use of NPK+FYM enhanced C and N in humic substances as compared to fallow. The functionality of the humic substances was reduced in the control, whereas integrated nutrient management increased the carboxylic (-COOH) and phenolic-OH groups. Hence, manuring along with fertilizers is the best way to maintain active humus in the soil over the years.

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# GENE SYSTEM GOVERNING STOMATAL NUMBER AND SPECIFIC LEAF WEIGHT IN BREAD WHEAT (*Triticum aestivum* L.)

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Gene effects were analysed using mean stomatal number and specific leaf weight of 12 populations, consisting of both parents ($P_1$ and $P_2$), $F_1$, $F_2$, first backcross generations ($BC_1$ and $BC_2$), second backcross generations ($B_{11}$, $B_{12}$, $B_{21}$, $B_{22}$) and backcross selfed generations ($B_1$s and $B_2$s) of four crosses involving three drought-tolerant and three drought-susceptible cultivars of *Triticum aestivum* L. to determine the nature of gene action governing stomatal number (SN) and specific leaf weight (SLW) through generation mean analysis in moisture stress ($E_1$) and moisture non-stress ($E_2$) environments. The digenic epistatic model was found to be inadequate for stomatal number and the additive–dominance model was found to be adequate for specific leaf weight in most of the crosses. Additive gene effects were predominant for SLW, while for SN both additive and dominance components of variance were important. Epistatic effects, particularly the additive × dominance (j) type of interaction, were present for both the characters. The duplicate type of epistasis was observed for stomatal number in the cross VL421/HS240 in the moisture stress environment. Significant heterosis was observed for the crosses Hindi 62/HS240 and VL421/HS240 over the standard check (SC) in the moisture stress environment ($E_1$) for both the characters. Genotype–environmental interactions and/or differential gene expression appeared to account for the different results found between environments. Hybridization systems, such as biparental mating and/or diallel selective mating, could be useful for the improvement of these traits, which would help in identifying drought-tolerant progenies.

**Key words:** *Triticum aestivum*, stomatal number, specific leaf weight, duplicate epistasis, heterosis

## Introduction

Among the cereals, wheat is an important crop for sustaining human life. At least 60 million ha of wheat is grown in marginal rainfed environments in developing countries. National average yields range from 0.8 to 1.5 t/ha,

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approximately 10 to 50% of their theoretical irrigated potential (Morris et al., 1991). To meet the demands projected for the future, the productivity level under drought conditions will have to be enhanced. However, the development of genotypes for dryland conditions requires an effective selection criterion/criteria and the positive response of the recombinants in segregating generations. Thus, the genetic improvement of wheat requires the exploitation of genetic variation for drought resistance and its utilization in breeding programmes. In the present study, two physiological parameters, stomatal number and specific leaf weight, were investigated, as these traits are very important drought-conferring characters. Stomatal frequency regulates the water loss in green parts of the plant and differences in stomatal behaviour appear to explain differences in drought resistance between species. A decrease in stomatal frequency reduces the transpiration rate (Miskin and Rasmuson, 1970). Parson (1979) advocated the use of stomatal frequency as a useful trait for drought tolerance. Thinner, wider leaves (i.e. with relatively low specific leaf weight) and a more prostrate growth habit help to increase ground cover, thus conserving soil moisture and potentially increasing radiation use efficiency (Richards, 1996). Information on the genetic architecture of these traits is scanty, so the present investigation was undertaken to understand the nature of gene action and to identify potential cross(es) for the development of drought-tolerant genotypes.

## Materials and methods

The experimental materials used for the present study consisted of twelve generations ($P_1$, $P_2$, $F_1$, $F_2$, $BC_1$, $BC_2$, $B_{11}$, $B_{12}$, $B_{21}$, $B_{22}$, $B_1$s and $B_2$s) generated from each of the four crosses, namely S4 × HPW89, Hindi 62 × HS240, VL421 × HS240 and VL421 × PBW175, involving three diverse drought-tolerant cultivars (Hindi 62, VL421 and S4), a drought-tolerant CIMMYT stock and three diverse drought-susceptible but high-yielding cultivars (HS240, PBW175 and HPW89), which are well suited to the agronomic conditions of the area under study. These twelve populations of each of the four crosses were evaluated in a compact family block design with three replications in moisture stress ($E_1$) and moisture non-stress ($E_2$) environments in the same cropping season, sown on 9$^{th}$ November, 2002. Each replicate was divided into four compact blocks. The four crosses, each consisting of twelve populations, were randomly allotted to the four blocks. All twelve generations were then randomly allotted to twelve plots within each block. The plots of various generations contained different numbers of rows, i.e. the non-segregating generations of different crosses (parents and $F_1$s) were grown in single rows 2 m in length per replication. The segregating $F_2$ generations of each of the crosses were grown in six rows; $BC_1$ and $BC_2$ in four rows; $B_1$s and $B_2$s in three rows and $B_{11}$, $B_{12}$, $B_{21}$ and $B_{22}$ in two rows in each of the replications. The inter-row and inter-plant distances were 23 cm and 5 cm, respectively. The data were recorded on 5 random plants of each parent and $F_1$, 20 plants of each first backcross generation, 40 plants of each $F_2$, 10 plants of each second backcross generation and 15 plants of each backcross selfed generation in each replication in both the environments. The present investigation was carried out at the experimental farm of the Department of Plant Breeding and Genetics, CSK HPAU, Palampur, situated at an elevation of about 1300 m amsl and at 32°6'N latitude and 76°3' longitude with a sub-temperate climate. The experiments were sown after one presowing irrigation with no further irrigation (rainfed conditions). The total precipitation received during the crop

season (Nov.–May) was 213.9 mm, with evaporation loss of 98 mm. However, the rainfall was erratic throughout the crop season, which resulted in moisture stress conditions (Fig. 1). The minimum and maximum temperatures varied from 3.6 to 17.9°C and 14.32 to 32.7°C, respectively. The humidity varied from 29.5% to 72.5% during the crop growing season.

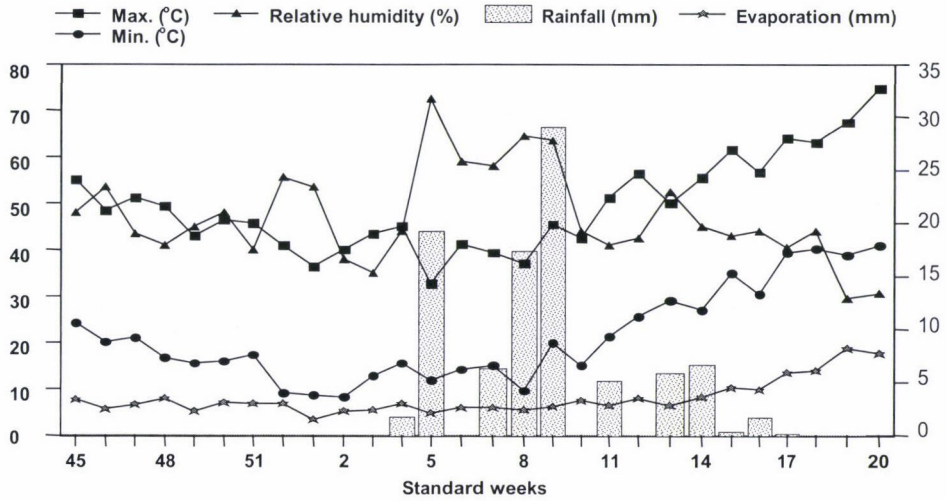



*Fig. 1.* Mean weekly weather data for the period November 2002 to May 2003

To determine the stomatal number the penultimate leaf of the second tiller was collected in the field and kept in blotting papers. The widest part of the leaf was sampled using a quick fix solution layer. The stomatal impressions were examined microscopically at 400× magnification, in five random microscopic fields. The number of stomata was counted for each field and averaged to calculate the mean.

Specific leaf weight was computed for the flag leaf of the second tiller as follows:

$$\text{Specific leaf weight} = \frac{\text{Leaf dry weight (mg)}}{\text{Leaf area (cm}^2\text{)}}$$

Standard statistical procedures were used to obtain means and variances for each environment separately (Snedecor and Cochram, 1968). When calculating variances, the replicate effect was eliminated from the total variances to obtain within-replicate variance. These variances were used to compute the standard error for each generation mean in each environment. Simple scaling tests (A, B, C and D) were carried out using the method of Mather (1949) and Hayman and Mather (1955), as well as the joint scaling test proposed by Cavalli (1952) to estimate genetic parameters with the 3-parameter non-epistatic model [(m), (d) and (h)] and the 6-parameter model, assuming digenic epistatic interaction [m, (d), (h), (i), (j) and (l)].

The estimates for gene effects were obtained by the weighted least square technique. Initially, twelve equations were developed by equating the observed generation means with their expectations in the presence of digenic interactions, as proposed by Hill (1966). Generation means and their expectations were weighted, appropriate weights being the reciprocals of the square standard errors. The twelve simultaneous equations thus obtained were solved by means of matrix inversions as follows:

$$M = J^{-1}S$$

where M = the column vector of the estimates of the parameter; S = the matrix of the score (right hand side); J = the information matrix; $J^{-1}$ = the inverse of the information matrix J (variance–covariance matrix).

The adequacy of the model was tested by predicting the twelve-generation mean from the estimate of each of the 3- and 6-parameter models by comparison of the weighted deviations of the observed and expected generation means in the form of a chi-square test with (n–p) d.f., which provides a test of the goodness of fit of a model. In this situation n is the number of generations and p is the number of parameters. The estimates of $\chi^2$(n–p) were obtained as

$$\chi^2 (n–p) = \Sigma(Qi–Ei)^2 \times wi$$

where Qi = is the observed mean of the $i^{th}$ generation; Ei = is the expected mean of the $i^{th}$ generation; wi = is the weight of the $i^{th}$ generation, calculated as:

$$wi = 1/V\,\bar{x} = 1/SE^2\,\bar{x}$$

In the digenic epistatic model the parameters estimated were: m = mean of all homozygous lines; (d) = additive gene effects pooled over all loci; (h) = dominance gene effects pooled over all loci; (i) = overall additive × additive epistatic gene effects; (j) = overall additive × dominance epistatic gene effects and (l) = overall dominance × dominance epistatic effects.

The magnitude of heterosis was estimated in relation to the better parent (BP) and the standard check (SC;VL421) under both the environments as follows:

$$1.\ \text{Heterosis over better parent (\%)} = \frac{\overline{F_1} - \overline{BP}}{BP} \times 100$$

$$2.\ \text{Heterosis over standard check (\%)} = \frac{\overline{F_1} - \overline{SC}}{\overline{SC}} \times 100$$

Test of significance:

$$\text{Heterosis for BP} = \frac{\overline{F_1} - \overline{BP}}{SE\,(H_1)} = t_1 \text{ calculated value}$$

$$\text{Heterosis for SC} = \frac{\overline{F_1} - \overline{SC}}{SE\,(H_2)} = t_2 \text{ calculated value}$$

The calculated values of 't' were compared with tabulated values for the error degrees of freedom at the P≤ 0.05 level of significance.

## Results

The mean performance of both the characters was considerably lower for almost all the generations under rainfed conditions than under irrigated conditions due to the inadequate availability of moisture under rainfed conditions (Table 1). The additive–dominance model was adequate for specific leaf weight in the cross Hindi62/HS240 in both the environments and for stomatal number in the same cross in the moisture stress environment ($E_1$). This model also showed its adequacy for SLW in the crosses VL421/HS240 and VL421/PBW175 and for SN in the cross S4/HPW89 in the non-stressed environment ($E_2$) (Tables 2 and 3), as indicated by the non-significant values of the simple scaling and $\chi^2$ tests. The non-significant value of the $\chi^2$ test for the digenic epistatic model showed its adequacy for SLW in the crosses S4/HPW89 and VL421/HS240 under $E_1$ and for SN in the crosses Hindi 62/HS240 and VL421/HS240 in the non-stressed environment ($E_2$). In some of the crosses, depending on the environment, a significant $\chi^2$ value was also observed for the 6-parameter model for both characters, indicating that there might be trigenic/higher order interactions present in these crosses. Significant negative

*Table 1*

Generation means and standard errors for stomatal number (SN) and specific leaf weight (SLW) in moisture stress ($E_1$) and non-stress ($E_2$) environments for four crosses of bread wheat

| Generations | S4 × HPW89 | | | | Hindi 62 × HS240 | | | |
|---|---|---|---|---|---|---|---|---|
| | SN | | SLW | | SN | | SLW | |
| | $E_1$ | $E_2$ | $E_1$ | $E_2$ | $E_1$ | $E_2$ | $E_1$ | $E_2$ |
| $P_1$ | 3.37 (±0.38) | 3.47 (±0.14) | 12.84 (±0.25) | 13.21 (±1.89) | 3.31 (±0.26) | 3.44 (± 0.04) | 8.08 (±0.36) | 9.97 (±0.22) |
| $P_2$ | 2.70 (±0.06) | 3.26 (±0.31) | 11.39 (±1.14) | 11.68 (±2.00) | 3.64 (±0.07) | 3.69 (±0.06) | 9.64 (±0.38) | 10.71 (±0.98) |
| $F_1$ | 2.97 (±0.20) | 3.13 (±0.01) | 11.76 (±1.22) | 12.96 (±0.72) | 2.98 (±0.10) | 3.37 (±0.08) | 10.84 (±0.08) | 9.74 (±0.56) |
| $F_2$ | 3.31 (±0.12) | 3.39 (±0.11) | 11.68 (±1.34) | 12.06 (± 0.89) | 3.25 (± 0.08) | 3.32 (±0.07) | 9.66 (±0.48) | 10.39 (±1.45) |
| $BC_1$ | 3.10 (±0.14) | 3.42 (±0.17) | 10.07 (±0.63) | 10.46 (±0.32) | 3.25 (±0.34) | 3.16 (±0.10) | 10.27 (±0.24) | 10.74 (±1.30) |
| $BC_2$ | 3.22 (±0.06) | 3.29 (±0.11) | 10.67(±0.37) | 11.82 (±0.61) | 3.35 (±0.18) | 3.44 (±0.16) | 10.19 (±1.30) | 10.22 (±0.62) |
| $B_{11}$ | 3.30 (±0.06) | 3.24 (±0.06) | 11.18 (±1.29) | 11.63 (±1.32) | 3.41 (±0.23) | 3.43 (±0.21) | 7.89 (±0.59) | 9.27 (±0.40) |
| $B_{12}$ | 3.22 (±0.11) | 3.22 (±0.13) | 13.00 (±1.24) | 13.37 (±1.57) | 2.90 (±0.39) | 3.38 (±0.14) | 10.18 (±0.29) | 10.28 (±0.76) |
| $B_{21}$ | 3.18 (±0.21) | 3.29 (±0.19) | 9.95 (±0.67) | 10.39 (±0.34) | 3.52 (±0.25) | 3.68 (±0.14) | 9.89 (±0.41) | 10.33 (±0.35) |
| $B_{22}$ | 2.90 (±0.06) | 3.22 (±0.17) | 11.22 (±0.64) | 12.57 (±0.17) | 3.39 (±0.11) | 3.39 (±0.19) | 10.53 (±0.27) | 10.04 (±0.42) |
| $B_1S$ | 2.96 (±0.10) | 3.24 (±0.13) | 11.12 (±0.37) | 13.03 (±0.69) | 3.55 (±0.23) | 3.59 (±0.09) | 9.55 (±0.72) | 9.97 (±0.09) |
| $B_2S$ | 3.26 (±0.30) | 3.28 (±0.16) | 9.76 (±0.82) | 10.17 (±0.17) | 3.71 (±0.16) | 3.40 (±0.17) | 10.48 (±0.40) | 10.22 (±0.67) |
| | VL421 × HS240 | | | | VL421 × PBW175 | | | |
| $P_1$ | 3.79 (±0.10) | 3.46 (±0.09) | 7.17 (0.79) | 9.14 (±0.68) | 3.79 (±0.10) | 3.46 (±0.09) | 7.17 (±0.79) | 9.14 (±0.68) |
| $P_2$ | 3.64 (±0.07) | 3.69 (±0.06) | 9.64 (±0.38) | 10.71 (±0.98) | 2.87 (±0.30) | 3.11 (±0.18) | 8.92 (±1.18) | 9.04 (±0.66) |
| $F_1$ | 3.16 (±0.07) | 3.73 (±0.04) | 9.18 (±0.12) | 10.16 (±0.09) | 2.81 (±0.34) | 3.20 (±0.10) | 8.60 (±0.51) | 9.38 (±0.08) |
| $F_2$ | 3.36 (±0.08) | 3.46 (±0.10) | 9.91 (±0.34) | 10.52 (± 0.49) | 3.11 (±0.07) | 3.33 (±0.21) | 8.41 (±0.65) | 9.36 (±0.10) |
| $BC_1$ | 3.58 (±0.12) | 3.58 (±0.16) | 9.89 (±0.46) | 10.25 (±0.52) | 3.60 (±0.20) | 3.38 (±0.03) | 7.94 (±0.87) | 9.55 (±0.53) |
| $BC_2$ | 3.47 (± 0.07) | 3.49 (±0.08) | 8.91 (±0.40) | 10.71 (±0.21) | 3.15 (± 0.17) | 2.98 (±0.02) | 8.36 (±0.14) | 9.70 (±0.08) |
| $B_{11}$ | 3.33 (±0.14) | 3.69 (±0.08) | 10.66 (± 1.31) | 10.42 (±0.43) | 3.22 (±0.35) | 3.33 (± 0.24) | 6.72 (±0.78) | 9.09 (± )0.46 |
| $B_{12}$ | 3.22 (±0.12) | 3.39 (±0.12) | 9.73 (± 1.46) | 10.06 (±0.79) | 2.84 (±0.14) | 3.20 (±0.12) | 7.72 (±0.51) | 9.29 (±0.31) |
| $B_{21}$ | 3.48 (±0.07) | 3.69 (±0.09) | 10.82 (±0.51) | 9.07 (±0.79) | 2.99 (±0.16) | 3.16 (±0.14) | 6.73 (±0.28) | 9.79 (±0.30) |
| $B_{22}$ | 3.89 (±0.06) | 3.56 (±0.18) | 8.77 (± 1.77) | 10.02 (±0.45) | 2.90 (±0.10) | 3.29 (±0.06) | 7.41 (±0.46) | 9.54 (±0.19) |
| $B_1S$ | 3.39 (±0.25) | 3.43 (±0.07) | 10.12 (±0.09) | 10.52 (±0.59) | 3.18 (±0.06) | 3.06 (±0.03) | 6.92 (±0.51) | 9.32 (±0.47) |
| $B_2S$ | 3.34 (±0.13) | 3.51 (±0.06) | 10.63 (± 1.27) | 10.82 (±0.26) | 3.22 (±0.06) | 3.30 (±0.19) | 6.95 (±0.73) | 9.09 (±0.47) |

gene effects of an additive nature (in a desirable direction for both the traits) were exhibited for SLW by the cross VL421/HS240 under $E_1$ and for SN by the crosses Hindi 62/HS240 and VL421/HS240 under $E_2$. Negative dominance gene effects were exhibited in the crosses S4/HPW89 and Hindi 62/HS240 under $E_2$ and $E_1$, respectively, for stomatal number, while no cross showed negative dominance gene effects for specific leaf weight.

The negative additive × additive (i) type of interaction was present for SLW in the cross VL421/HS240 under $E_1$, while no cross showed the negative (i) type of interaction for SF. The negative additive × dominance (j) type of interaction was found for SLW and SN in the crosses VL421/PBW175 and S4/HPW89, respectively, in a moisture stress environment. Only the cross VL421/HS240 under $E_1$ showed the dominance × dominance (l) type of interaction along with the duplicate type of epistasis for stomatal number.

*Table 2*
Estimates of Scaling tests for stomatal number and specific leaf weight

| Crosses | Env. | A | B | C | D | $\chi^2$ (9 d.f.) |
|---|---|---|---|---|---|---|
| Stomatal number | | | | | | |
| $S_4$ × HPW89 | $E_1$ | –0.14±0.52 | 0.77±0.24 | 1.25±0.73 | 0.31±0.28 | 102.45* |
| | $E_2$ | 0.25±0.37 | 0.19±0.38 | 0.58±0.55 | 0.07±0.28 | 5.15 |
| Hindi 62 × HS240 | $E_1$ | 0.22±0.73 | 0.09±0.37 | 0.11±0.45 | –0.10±0.41 | 9.20 |
| | $E_2$ | –0.50*±0.22 | –0.18±0.33 | –0.60±0.33 | 0.04±0.23 | 12.32 |
| VL421 × HS240 | $E_1$ | 0.21±0.27 | 0.13±0.47 | –0.32±0.37 | –0.33±0.21 | 75.51* |
| | $E_2$ | –0.04± 0.33 | –0.43*±0.18 | –0.78±0.40 | –0.15±0.26 | 24.11* |
| VL421 × PBW175 | $E_1$ | 0.60±0.53 | 0.62±0.56 | 0.17±0.80 | –0.52±0.20 | 19.54* |
| | $E_2$ | 0.04±0.15 | –0.35±0.21 | 0.34±0.89 | 0.30±0.42 | 55.81* |
| Specific leaf weight | | | | | | |
| $S_4$ × HPW89 | $E_1$ | –4.45*±1.76 | –1.82±1.82 | –1.04±5.99 | 2.62±2.77 | 18.76* |
| | $E_2$ | –5.25*±2.12 | –1.00±2.46 | –2.5±4.74 | 1.83±1.91 | 32.80* |
| Hindi62 × HS240 | $E_1$ | 1.62±1.44 | –0.09±2.62 | –0.76±2.39 | –1.14±1.64 | 14.66 |
| | $E_2$ | 1.77±2.67 | –0.003±1.67 | 1.42±5.10 | –0.17±3.24 | 3.38 |
| VL421 × HS240 | $E_1$ | 3.42*±1.21 | –1.00±0.88 | 4.48*±1.62 | 1.03±0.90 | 26.61* |
| | $E_2$ | 1.19±1.23 | 0.54±1.07 | 1.89±2.29 | 0.07±1.12 | 8.68 |
| VL421 × PBW175 | $E_1$ | 0.11±1.98 | –0.81±1.32 | 0.33±3.14 | 0.51±1.58 | 25.35* |
| | $E_2$ | 0.59±1.26 | 0.98±0.68 | 0.46±1.04 | –0.54±0.57 | 6.85 |

$E_1$ = Moisture stress; $E_2$ = Non-stressed environment; Significant at the *P≤0.05 level

*Table 3*
Estimates of genetic parameters for stomatal number and specific leaf weight

| Crosses | Env. | m | d | h | i | j | l | Epi. | $\chi^2$ (6 d.f.) |
|---|---|---|---|---|---|---|---|---|---|
| Stomatal number | | | | | | | | | |
| $S_4$ × HPW89 | $E_1$ | 3.36*±0.18 | 0.36*±0.09 | `0.04±0.68 | –0.28±0.15 | –1.17*±0.30 | –0.57±0.63 | | 16.42* |
| | $E_2$ | 3.38*±0.06 | –0.04±0.06 | –0.25*±0.07 | – | – | – | | – |
| Hindi 62 × HS240 | $E_1$ | 3.60*±0.08 | –0.05±0.08 | –0.54*±0.13 | – | – | – | | – |
| | $E_2$ | 3.90*±0.28 | –0.12*±0.03 | –1.47±0.77 | –0.32±0.27 | –0.06±0.27 | 0.93±0.53 | | 8.36 |
| VL421 × HS240 | $E_1$ | 2.61*±0.28 | –0.02±0.05 | 2.45*±0.68 | 1.01*±0.27 | 0.96*±0.20 | –1.78*±0.44 | D | 44.30* |
| | $E_2$ | 3.26*±0.19 | –0.11*±0.05 | 0.49±0.56 | 0.35±0.20 | 0.49*±0.24 | –0.44±0.39 | | 8.11 |
| VL421 × PBW175 | $E_1$ | 3.04*±0.30 | 0.32*±0.11 | 0.58±1.11 | 0.38±0.32 | –0.67±0.40 | –0.91±1.00 | | 13.54* |
| | $E_2$ | 2.45*±0.13 | 0.10±0.08 | 1.26*±0.36 | 0.87*±0.15 | 0.54*±0.19 | –0.49±0.30 | | 12.93* |
| Specific leaf weight | | | | | | | | | |
| $S_4$ × HPW89 | $E_1$ | 11.94*±1.51 | 1.20*±0.49 | –6.13±4.47 | –0.29±1.52 | –2.79±1.63 | 6.97±3.76 | | 5.25 |
| | $E_2$ | 10.53*±0.96 | 1.95*±0.80 | –0.79±3.50 | 0.43±1.02 | –4.34±2.29 | 4.77±3.02 | | 24.88* |
| Hindi 62 × HS240 | $E_1$ | 9.33*±0.21 | –0.35±0.22 | 1.51*±0.25 | – | – | – | | – |
| | $E_2$ | 10.21*±0.20 | –0.33±0.21 | –0.33±0.49 | – | – | – | | – |
| VL421 × HS240 | $E_1$ | 11.44*±0.96 | –1.03*±0.39 | –2.40±2.86 | –2.55*±1.10 | 3.28*±1.42 | 0.18±1.96 | | 11.49 |
| | $E_2$ | 10.57*±0.21 | –0.46±0.24 | –0.40±0.24 | – | – | – | | – |
| VL421 × PBW175 | $E_1$ | 6.58*±1.43 | 0.64±0.47 | 2.90±3.68 | –0.16±1.47 | –3.30*±1.39 | 1.04 | | 17.31* |
| | $E_2$ | 8.25*±0.60 | –0.20±0.35 | 3.30*±1.68 | – | – | – | | – |

$E_1$ = Moisture stress; $E_2$ = Non-stressed environment; Significant at the *P≤0.05 level

Significant negative heterosis (in the desirable direction) over BP was observed in the cross VL421/HS240 under $E_1$ and over SC in all the crosses in both the environments, except VL421/PBW175 under $E_2$ for SN, while no cross showed significant negative desirable heterosis for specific leaf weight.

## Discussion

Significant differences were observed between the generation means for stomatal number and specific leaf weight in all four crosses, which revealed the presence of genetic diversity for these characters in the materials (Table 4). Relatively consistent differences between drought-resistant and drought-susceptible cultivars for stomatal number and specific leaf weight indicated that a selection programme for high or low stomatal number and specific leaf weight would be effective. Hybrids from crosses of these varieties can be expected to produce desirable segregants for stomatal number and specific leaf weight under drought stress conditions. However, the existence of high genotype–environment interactions, involving differences in gene expression at various stages, should also be taken into account (Jana, 1975; Mehta et al., 1992). The genetic architecture of the four crosses for both the characters, however, differed from each other. There was wide variation not only with regard to the nature and magnitude of gene action, but also in their sensitivity to variation in the environmental conditions.

The presence of additive gene effects for stomatal number in the crosses Hindi 62/HS240 and VL421/HS240 under $E_2$ and for specific leaf weight in the cross VL421/HS240, along with additive × additive type interaction under $E_1$, indicated the effectiveness of early selection for the improvement of these traits in the above crosses. The dominance gene effects observed in the crosses S4/HPW89 and Hindi 62/HS240 under $E_2$ and $E_1$, respectively, and the j type of interaction in the cross S4/HPW89 for SN and in the cross VL421/PBW175 for SLW under $E_1$ suggested that selection should be deferred to later generations. Duplicate epistasis coupled with the l type of interaction was observed in the cross VL421/HS240 under $E_1$ for stomatal number, suggesting that selection would be effective if dominance and epistatic effects were reduced after a few generations of selfing and/or intermating in early generations. (Nayeem and Garshin, 1990) also reported additive gene effects for stomatal number, while Islam et al. (1998) reported non-additive gene effects. Both the additive–dominance and digenic epistatic models were found to be adequate for both the characters, though some crosses also showed significant values of the $\chi^2$ test for the digenic epistatic model, indicating that there might be trigenic/higher order linkage among the genes for these characters. Further study will be required to establish the higher order interactions. It was also found that for SLW the h and l components were non-significant. On the basis of heterosis studies, two crosses, namely Hindi 62/HS240 and VL421/HS240, were adjudged to be best for exploitation.

*Table 4*
Estimates of heterosis in moisture stress ($E_1$) and non-stress ($E_2$) environments

| Crosses | Percentage deviation from BP | | Percentage deviation from SC | |
|---|---|---|---|---|
| | $E_1$ | $E_2$ | $E_1$ | $E_2$ |
| Stomatal number | | | | |
| S4 × HPW 89 | 10.00 | –3.99 | –27.61* | –9.53* |
| Hindi 62 × HS240 | –9.97 | –2.03 | –13.87* | –11.08* |
| VL 421 × HS240 | –13.19* | 7.80* | –16.62* | –7.80* |
| VL 421 × PBW 175 | –2.09 | 2.89 | –25.85* | –7.51 |
| Specific leaf weight | | | | |
| S4 × HPW 89 | –8.41 | 1.89 | 64.01* | 41.79* |
| Hindi 62 × HS240 | 12.45* | –9.06 | 51.18* | 6.56 |
| VL 421 × HS240 | –4.77 | –5.14 | 28.03* | 11.16 |
| VL 421 × PBW 175 | –3.59 | 3.16 | 19.94 | 2.63 |

Significant at the *P≤0.05 level; BP = Better parent; SC = standard check

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# EVALUATION OF BLUE-GREEN ALGAL INOCULATION ON SPECIFIC SOIL PARAMETERS

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The impact of nitrogen-fixing blue-green algal (BGA) strains, namely *Anabaena variabilis, Aulosira fertilissima, Nostoc muscorum* and *Tolypothrix tenuis,* was studied at different levels of nitrogen fertilizer on specific soil parameters such as microbial populations, pH, EC, redox potential, chlorophyll, dehydrogenase and nitrogenase activity under a rice crop. The inoculation of the soil with BGA strains resulted in higher microbial populations (BGA, bacteria, fungi and actinomycetes) and had a significant influence on redox potential. A significant increase in soil chlorophyll, dehydrogenase and nitrogenase activity was observed during crop growth due to BGA application.

**Key words:** BGA, redox potential, soil chlorophyll, dehydrogenase, nitrogenase

## Introduction

The use of nitrogen-fixing blue-green algae (BGA)/cyanobacteria for rice has great significance in increasing crop yields (Roger et al., 1993). These have been identified as an alternative source of nitrogen and their role in rice fields has been well demonstrated and widely documented (Venkataraman, 1981). A build-up of organic matter due to algal inoculation in rice soil has also been reported (De and Sulaiman, 1950; Das et al., 1991). These organisms are also known to secrete growth-promoting substances, solubilize insoluble phosphates, improve the fertilizer use efficiency of crop plants and amend the physical and chemical properties of soil (Goyal, 1993). Hence the present work has been focused to understand the influence of $N_2$-fixing blue-green algae on the microbial population, redox potential, specific enzymes and chlorophyll content of the soil under rice crops.

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## Materials and methods

Blue-green algal strains, namely *Anabaena variabilis, Aulosira fertilissima, Nostoc muscorum* and *Tolypothrix tenuis,* were applied separately or with different levels of nitrogen fertilizer (0, 60, 120 kg N $ha^{-1}$) to a rice crop (variety Pusa Basmati-1). The experiment was laid out in a complete randomized design (CRD) with three replications for each treatment and was repeated for three cropping seasons. Inoculation with blue-green algae was given @ 25 μg chl $kg^{-1}$ soil over the surface of standing water, 7 days after transplanting. Urea was applied in split doses of 50:25:25, as basal, and at 30 and 60 DAT (days after transplanting). Phosphate and potash were applied as basal dressing in the form of SSP and MOP @ 50 kg $ha^{-1}$.

Soil samples were taken 45 and 90 DAT. These were air dried, powdered and passed through a 2 mm sieve for the analysis of microbial populations (BGA, bacteria, fungi and actinomycetes) and dehydrogenase activity. BG-11 medium was used for the determination of blue-green algal populations (Stanier et al., 1971), while soil extract agar medium (Parkinson et al., 1971), Martin's Rose Bengal agar medium (Martin, 1950) and starch casein medium (Kuster and Williams, 1964) were used for the enumeration of bacteria, fungi and actinomycetes, respectively. The redox potential (Eh) of the soil sample was measured 3 cm below the soil water interface using a portable redox meter fitted with a compound platinum and calomel electrode (Saha et al., 1982).

For the estimation of soil chlorophyll, preweighed soil cores were extracted in an acetone: dimethyl sulphoxide (DMSO) mixture and the concentrations were determined from absorbance readings taken at 750, 663, 645 and 630 nm (Tsujimura et al., 2000). The dehydrogenase activity was determined by the 2,3,5-triphenyl tetrazolium chloride (TTC) reduction method (Klein et al., 1971) by measuring the absorbance of triphenyl formazon (TPF) at 485 nm. A standard calibration curve was prepared from triphenyl formazan and the activity was expressed as μl $H_2$ released $g^{-1}$ soil $day^{-1}$. Soil nitrogenase activity was examined by acetylene reduction assay (ARA) using gas chromatography (GC 1000, Chemito model) with a N column (Prasanna et al., 2003).

*Statistical analysis*

The data were subjected to statistical analysis using the M-STATC package according to factorial CRD. Duncan's Multiple Range Test (DMRT) was employed to compare the mean performances of strains for the specific parameters under study. Values denoted by the same superscript are not significantly different.

## Results and discussion

There was an enhancement in the soil algal population with the application of blue-green algal strains at the midcrop (45 DAT) and harvest stage (90 DAT) of crop growth (Table 1). The results obtained are in agreement with earlier studies, where the number of blue-green algal species inoculated increased on flooded brown earth silt loam (Rao and Burns, 1990), though by day 147 the number of introduced BGA was no different from that on non-inoculated soils. The bacterial population was highest and contributed significantly to the total microbial flora, thus revealing that even if artificial inoculation in the form of blue-green algal application was given, the maximum contribution to the total microbial flora was through the bacterial population. The fungal and actinomycetes populations were also enhanced due to blue-green algal inoculation at three different levels of nitrogenous fertilizer (Table 1).

*Table 1*
Influence of blue-green algal inoculation on specific soil microbial flora under different levels of nitrogenous fertilizers

| Strains | N level | Algal population (cfu×$10^3$g$^{-1}$ dry soil) | | Bacteria (cfu×$10^6$g$^{-1}$ dry soil) | | Fungi (cfu×$10^2$g$^{-1}$ dry soil) | | Actinomycetes (cfu×$10^4$g$^{-1}$ dry soil) | |
|---|---|---|---|---|---|---|---|---|---|
| | | 45 DAT | 90 DAT | 45 DAT | 90 DAT | 45 DAT | 90 DAT | 45 DAT | 90 DAT |
| $S_0$ | $N_0$ | 8.73 | 4.8 | 35.67 | 36.67 | 16.67 | 12.67 | 1.33 | 1.67 |
| | $N_{60}$ | 10.33 | 3.13 | 40.0 | 41.33 | 21.0 | 18.0 | 2.33 | 3.67 |
| | $N_{120}$ | 15.0 | 3.47 | 51.33$^{b}$ | 50.33$^{b}$ | 25.0 | 24.33 | 3.33 | 4.67 |
| $S_1$ | $N_0$ | 10.13 | 6.17$^{a}$ | 38.67 | 44.0 | 23.67 | 18.0 | 3.33 | 4.67 |
| | $N_{60}$ | 14.67 | 4.2 | 49.67$^{bc}$ | 44.67 | 26.67 | 21.0 | 6.67 | 6.67 |
| | $N_{120}$ | 21.67$^{b}$ | 4.33 | 58.0$^{a}$ | 53.33$^{a}$ | 30.0$^{b}$ | 26.0$^{b}$ | 8.33$^{bc}$ | 7.67$^{ab}$ |
| $S_2$ | $N_0$ | 7.33 | 5.5$^{c}$ | 35.0 | 42.0 | 21.33 | 17.0 | 3.0 | 3.67 |
| | $N_{60}$ | 13.0 | 4.1 | 50.67$^{b}$ | 44.33 | 25.0 | 19.33 | 6.67 | 5.0 |
| | $N_{120}$ | 19.0 | 4.3 | 55.67$^{a}$ | 52.0$^{ab}$ | 30.0$^{b}$ | 28.0$^{a}$ | 10.0$^{a}$ | 6.33 |
| $S_3$ | $N_0$ | 7.67 | 6.07$^{ab}$ | 37.0 | 41.0 | 23.0 | 17.67 | 1.67 | 4.33 |
| | $N_{60}$ | 14.0 | 4.77 | 48.0 | 45.67 | 26.0 | 22.0 | 7.0 | 5.67 |
| | $N_{120}$ | 20.0$^{bc}$ | 4.2 | 56.0$^{a}$ | 52.67$^{a}$ | 28.67$^{bc}$ | 28.33$^{a}$ | 9.67$^{ab}$ | 7.33$^{abc}$ |
| $S_4$ | $N_0$ | 7.33 | 5.97$^{ab}$ | 36.33 | 41.33 | 23.0 | 17.0 | 1.33 | 3.67 |
| | $N_{60}$ | 13.33 | 4.77 | 49.33$^{bc}$ | 41.67 | 25.67 | 23.0 | 3.67 | 4.67 |
| | $N_{120}$ | 25.0$^{a}$ | 4.5 | 56.33$^{a}$ | 51.67$^{ab}$ | 39.33$^{a}$ | 27.33$^{ab}$ | 5.33 | 8.0$^{a}$ |
| $CD_{P=0.05}$ | N × S | 1.75 | 0.05 | 2.01 | 1.79 | 2.0 | 1.49 | 0.43 | 1.04 |

DMRT grouping in descending order, with the highest denoted by the letter a; $S_0$ = Without inoculation; $S_1$ = *Anabaena variabilis*; $S_2$ = *Aulosira fertilissima*; $S_3$ = *Nostoc muscorum:* $S_4$ = *Tolypothrix tenuis*

Unlike chemical N fertilizers, blue-green algae neither contaminate the environment nor consume the photosynthates of rice plants (Liu, 1979). Earlier studies have indicated a negative correlation between the concentration of inorganic nitrogen fertilizer and blue-green algal abundance (Quesada and Fernandez-Valiente, 1996). If certain aerobic and microaerophilic nitrogen-fixing organisms such as *Azotobacter*, *Azospirillum*, *Pseudomonas* and others are inoculated along with blue-green algae, they can make use of the excess oxygen and easily oxidisable carbonaceous metabolites liberated by BGA for their respiration and as a source of energy, respectively (Mandal et al., 1999). This process may compensate for the possible loss of nitrogen through denitrification and also augment atmospheric nitrogen addition to the soil. In a pot culture experiment with *Tolypothrix tenuis* as the inoculant, Ibrahim et al. (1971) observed an increase in the total microbial population, especially the number of nitrifiers, namely the genera *Azotobacter* and *Clostridium,* when a mixture of blue-green algal inocula was used (Rao and Burns, 1990). It was observed that there was an 8-fold increase in bacterial numbers 13 weeks after inoculation, while the increase was only 2–8-fold after 21 weeks. Similarly, Rogers and Burns (1994) recorded 500-fold, 16-fold and 48-fold increases in the bacterial, fungal and actinomycetes populations, respectively, in the treatments inoculated with *Nostoc muscorum* compared with the non-inoculated control.

The inoculation of the soil with blue-green algae increased the redox potential, probably due to the direct influence of increased ionic strength and the biological activities of soil microbes, in comparison to uninoculated control soils (Saha et al., 1982). The redox potential was highest in the presence of *Anabaena variabilis* at the harvest stage (Fig. 1). Blue-green algae are aerobic photosynthetic organisms and evolve oxygen during photosynthesis through photosystem II. As a result, when they grow in rice fields they make the standing water highly oxygenated (Harrison and Aiyer, 1913). The profuse growth of BGA may increase the oxygen concentration, possibly to as much as 10–12 μg $g^{-1}$ (Lakshmanan et al., 1994). This has an important beneficial effect in rice fields, where continuous waterlogging creates intense reducing conditions, with redox potential values falling below –200 mV (Ponnamperuma, 1972).

Soil cores from the $N_{120}$ + BGA treatments exhibited increased soil chlorophyll over the control. The highest soil chlorophyll content was recorded for *Tolypothrix tenuis* + $N_{120}$ with a chlorophyll content more than 85% greater than in the –N treatment, where no biofertilizer inoculum was provided at 45 DAT. At 90 DAT, *Anabaena variabilis* inoculation combined with $N_{120}$ showed the highest soil chlorophyll, followed by *Tolypothrix tenuis* inoculation at a similar level of N fertilizer. If chlorophyll *a* is properly extracted from the soil, the biomass estimated by pigment extraction techniques reflects the quantity of active cells at the time of sampling, since resting cells have little chlorophyll (Coleman, 1983).

The dehydrogenase activity seemed to follow the trend reported for total microbial biomass, as reported in other studies (Chander et al., 1997). DMRT analysis indicated that the highest dehydrogenase activity was observed after *Tolypothrix tenuis* inoculation in the presence of $N_{120}$ at 45 DAT. At harvest, the highest dehydrogenase activity was observed for *Anabaena variabilis* inoculation at $N_{120}$, followed by *Tolypothrix tenuis* inoculation at the same level of nitrogenous fertilizer (Table 2).

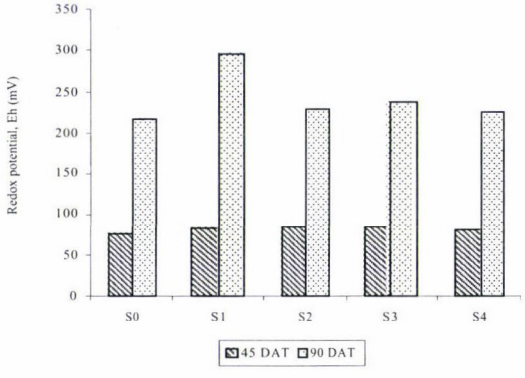



*Fig 1.* Influence of blue-green algal inoculation on soil redox potential ($S_0$ = Without inoculation; $S_1$ = *Anabaena variabilis*; $S_2$ = *Aulosira fertilissima*; $S_3$ = *Nostoc muscorum*; $S_4$ = *Tolypothrix tenuis*

*Table 2*
Influence of blue-green algal inoculation on specific soil properties under different levels of nitrogenous fertilizer

| Strains | N level | Soil chlorophyll (μg $g^{-1}$) | | Soil dehydrogenase activity (μl $H_2$ $g^{-1}$ $day^{-1}$) | | Soil nitrogenase activity (n mol $C_2H_4$ $g^{-1}$ $h^{-1}$) | |
|---|---|---|---|---|---|---|---|
| | | 45 DAT | 90 DAT | 45 DAT | 90 DAT | 45 DAT | 90 DAT |
| $S_0$ | $N_0$ | 23.34 | 6.87 | 33.14 | 9.75 | 0.55 | 0.35 |
| | $N_{60}$ | 30.92 | 12.71 | 43.9 | 18.04 | 0.78 | 0.04 |
| | $N_{120}$ | 48.97 | 39.2 | 69.53 | 55.66 | 0.82 | 0.06 |
| $S_1$ | $N_0$ | 64.9 | 21.67 | 92.16 | 30.77 | $9.08^{c}$ | $0.3^{bc}$ |
| | $N_{60}$ | 83.48 | 48.64 | 118.54 | 69.07 | $12.40^{b}$ | $0.44^{a}$ |
| | $N_{120}$ | $114.71^{b}$ | $65.41^{a}$ | $162.89^{b}$ | $92.88^{a}$ | $16.69^{a}$ | $0.32^{a}$ |
| $S_2$ | $N_0$ | 43.36 | 11.89 | 61.58 | 16.88 | 4.26 | 0.15 |
| | $N_{60}$ | 76.01 | 31.25 | 107.94 | 44.38 | 3.4 | 0.25 |
| | $N_{120}$ | 87.89 | 44.38 | 124.8 | 63.01 | 4.48 | 0.17 |
| $S_3$ | $N_0$ | 51.45 | 19.13 | 73.06 | 27.16 | 2.74 | 0.18 |
| | $N_{60}$ | 58.12 | 41.95 | 82.51 | 59.57 | $17.4^{a}$ | 0.15 |
| | $N_{120}$ | $107.27^{c}$ | $54.25^{c}$ | $152.33^{c}$ | $77.04^{c}$ | 7.34 | 0.25 |
| $S_4$ | $N_0$ | 39.21 | 8.06 | 55.69 | 11.44 | 0.96 | 0.1 |
| | $N_{60}$ | 65.41 | 26.03 | 92.88 | 36.97 | $9.62^{c}$ | $0.29^{bc}$ |
| | $N_{120}$ | $162.59^{a}$ | $61.39^{b}$ | $230.87^{a}$ | $87.18^{b}$ | 5.23 | 0.1 |
| $CD_{P=0.05}$ | N × S | 5.77 | 3.61 | 8.19 | 5.13 | 1.15 | 0.59 |
| | N | * | * | * | * | * | * |
| | S | * | * | * | * | * | * |

DMRT grouping in descending order, with the highest denoted by the letter a $S_0$ = Without inoculation; $S_1$ = *Anabaena variabilis*; $S_2$ = *Aulosira fertilissima*; $S_3$ = *Nostoc muscorum*; $S_4$ = *Tolypothrix tenuis*

The nitrogenase activity (acetylene reduction assay) of soil cores at 45 DAT was maximum for *Nostoc muscorum* inoculation at $N_{60}$, which was at par with the activity observed for *Anabaena variabilis* inoculation at $N_{120}$. However, at 90 DAT the highest nitrogenase activity was observed at 60 kg N $ha^{-1}$, followed by 120 kg N $ha^{-1}$ with *Anabaena variabilis,* which was at par with the activity recorded at $N_0$ with *Anabaena variabilis* application and at $N_{60}$ with *Tolypothrix tenuis* application (Table 2). The enhancement of the algal population corresponded with the increase in nitrogenase activity when the soils were artificially inoculated with blue-green algal strains. Mekonnen et al. (2002) observed that increasing the level of nitrogenous fertilizer decreased the nitrogenase activity, which is in accordance with the present study. The nitrogenase activity in the soil core was higher at 45 DAT than at harvest. In the rhizosphere of rice variety IR 26, acetylene reductase activity reached a maximum value at the heading stage, followed by a rapid decrease (Lee and Watanabe, 1977). When blue-green algal blooms were present, nitrogen fixation contributed 2 kg N $ha^{-1}$ $day^{-1}$ and the nitrogenase activity assayed at midday was from one to three orders of magnitude higher in BGA-covered soils in comparison to bare soil (Quesada et al., 1997). Although indigenous algal flora has greater competitive ability over inoculated strains, inoculum applied to the soil surface might benefit from more light and grow better than the indigenous flora mixed with the soil, as the growth and germination of these organisms is generally photo-dependent (Reddy, 1983).

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# CO-INOCULATION OF NITROGEN-FIXING AND PHOSPHATE-SOLUBILIZING BACTERIA TO PROMOTE GROWTH, YIELD AND NUTRIENT UPTAKE IN CHICKPEA

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A total of 32 bacterial isolates including *Mesorhizobium* (N=10), *Azotobacter* (N=12) and phosphate-solubilizing bacteria (N=10) were isolated and tested for siderophore, HCN, ammonia, indole acetic acid production and phosphate solubilization *in vitro*. The bacterial cultures were positive for siderophore, HCN and ammonia. Among the isolates, *M. ciceri* RC3 and *A. chroococcum* A4 displayed 35 and 14 μg $ml^{-1}$ of IAA, respectively, whereas *Bacillus* produced 19 (*Bacillus* PSB1) and 17 μg $ml^{-1}$ (*Bacillus* PSB10) of IAA in Luria Bertani broth. The diameter of the P solubilization zone varied between 4 (*Bacillus* PSB1) and 5 mm (*Bacillus* PSB10) and a considerable amount of tricalcium phosphate (7 and 8 μg $ml^{-1}$ by *Bacillus* PSB1 and *Bacillus* PSB10, respectively) was released in liquid medium, with a concomitant drop in pH. The effects of $N_2$-fixing and PS bacteria on the growth, chlorophyll content, seed yield, grain protein and N uptake of chickpea plants in field trials varied considerably between the treatments. Nodule number and biomass were significantly greater at 90 days after sowing (DAS), decreasing by 145 DAS. Seed yield increased by 250% due to inoculation with *M. ciceri* RC3 + *A. chroococcum* A4 + *Bacillus* PSB10, relative to the control treatment. Grain protein content ranged from 180 (*Bacillus* PSB1) to 309 ng $g^{-1}$ (*M. ciceri* RC3 + *A. chroococcum* A4 + *Bacillus* PSB10) in inoculated chickpea. The N contents in roots and shoots differed considerably among the treatments.

**Key words**: nitrogen-fixing organisms, phosphate-solubilizing bacteria, chickpea, N uptake

## Introduction

Phosphorus is one of the major plant nutrients limiting plant growth, although it is available both in organic and inorganic forms. Most agronomic soils contain large amounts of P, a considerable part of which has accumulated as a consequence of the consistent and excessive application of P fertilizers. However, a major portion of the P is fixed as insoluble forms soon after its

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application and becomes unavailable to the plants (Rodriguez and Fraga, 1999). Phosphorus in soils is immobilized or becomes less soluble either by absorption, chemical precipitation or both. In acid soils, it is fixed as oxides and hydroxides of Al and Fe (Norrish and Rosser, 1983), while in calcareous soils it is fixed mainly by Ca (Lindsay et al., 1989). The application of P fertilizers is therefore recommended to circumvent the P deficiency in soils. Microorganisms capable of solubilizing the insoluble inorganic P under such conditions and making it available to crop plants are referred to as phosphate-solubilizing microorganisms (PSM). Among the PSM, *Bacillus* and *Pseudomonas* (Illmer and Schinner, 1992) are the most important genera having PS activity. It has been reported that certain strains of *Rhizobium* can also solubilize inorganic P (Halder and Chakrabarty, 1993). Although the PS organisms and their activities remain concentrated in the rhizospheres of crop plants (Vazquez et al., 2000), they mediate important soil processes such as nutrient mobilization, decomposition, mineralization, release of nutrients and $N_2$ fixation and denitrification. The ability of PSM to solubilize insoluble P has been quantitatively investigated under *in vitro* conditions by several workers (Singal et al., 1994; Maliha et al., 2004). The solubilization of inorganic P by PSM is generally believed to be due to the production of organic acids (Whitelaw et al., 1999; Maliha et al., 2004) or the formation of soluble complexes with metal ions associated with insoluble P (Omar, 1998).

The beneficial effects of PSM either alone (Richardson et al., 2001) or in combination with other rhizospheric microorganisms (Zaidi et al., 2004) on legumes have been reported. During the intergeneric interaction, $N_2$-fixing bacteria provide N to the plant and consequently improve the N status of the soil, while PS bacteria solubilize the insoluble P and make it available to the crops (Gupta, 2004). In addition, the PS bacteria also release growth-promoting substances (Sattar and Gaur, 1987) that augment crop productivity. Therefore, for agronomic utility, the inoculation of the plants with rhizospheric microorganisms is necessary to take advantage of their beneficial properties for yield enhancement. However, information on the response of chickpea to dual or triple seed inoculation with symbiotic bacteria (*Mesorhizobium*), asymbiotic bacteria (*Azotobacter chroococcum*) and PS bacteria is scarce. The present study evaluates the effect of these cultures on the vitality, yield, foliage chlorophyll content and N uptake of chickpea (*Cicer arietinum* L.) grown under field conditions.

## Materials and methods

*Mesorhizobium* strains were isolated from nodules of chickpea, while *Azotobacter chroococcum* and PS bacteria were isolated from the rhizospheric soils of mustard (*Brassica campestris* L.) and tomato (*Lycopersicum esculentum* L.), grown in the experimental fields of the Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, using standard methods (Holt et al., 1994). *Mesorhizobium* sp. and *Azotobacter chroococcum* were isolated using the serial dilution plate technique on solid yeast extract mannitol and Ashby medium, respectively and were maintained on the same medium until used.

*Phosphate solubilization on solid and liquid medium*

The PS bacteria were isolated from rhizospheric soils using the dilution plate technique and tested for their PS activity on Pikovskaya medium (Pikovskaya, 1948). The plates were incubated at 28 ± 2°C for five days and observed for halo formation. Colonies forming a clear halo around them, indicating P solubilization, were counted and further used to determine the relative P solubilizing efficiency (RPSE) in liquid culture medium. For the quantitative measurement of P, 100 mL of Pikovskaya broth containing tricalcium phosphate (TCP) was inoculated with 1 ml of culture ($10^8$ cells/ml) and incubated for five days on a shaker (120 rpm) at 28 ± 2°C. The culture broth was centrifuged (15,000 rpm) for 30 min and the amount of water-soluble P released into the supernatant was estimated by the chlorostannous-reduced molybdophosphoric acid blue method (King, 1932; Jackson, 1967). The change in pH following TCP solubilization was also measured. Each experiment was repeated twice after several subcultures of the PS bacteria. Bacterial isolates showing greater solubilization on both solid and liquid medium and the persistence of PS activity after several subcultures were the criteria for choosing efficient PS strains.

*Bioassay of indole acetic acid*

The quantitative analysis of indole-3-acetic acid was performed by the method suggested by Gordon and Weber (1951) and later modified by Brick et al. (1991). Bacterial isolates were grown in Luria Bertani broth (g/l: tryptone 10; yeast extract 5; NaCl 10 and pH 7.5). A hundred ml of Luria Bertani broth amended with tryptophan (20, 60 and 100 μg $ml^{-1}$) was inoculated with 1 ml of culture (x $10^8$ cells/ml) and incubated for 24 h at 28°C on a rotary shaker (125 rpm). After 24 h, 5 ml of each culture was centrifuged (10,000 rpm) for 15 min and 2 ml of Salkowsky reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid) was added to 2 ml of supernatant and incubated at 28°C in the dark for 1 h. The IAA concentration was determined using a spectrophotometer (λ 540 nm) against a standard curve. The experiments were conducted three times at different time intervals.

In vitro *production assay of siderophore, HCN and ammonia*

The analysis of siderophore production by the bacterial isolates was performed following the chrome azurol S (CAS) method of Alexander and Zuberer (1991). For each bacterial isolate, 100 μl of inoculum was dropped in the centre of Petri dishes (8.5 cm diameter) containing 30 ml CAS agar. The dishes were incubated at 28°C for four days and were observed daily. The discoloration of the medium (blue to orange) indicated siderophore production by the bacterial isolates. Isolates positive for siderophore were screened and re-inoculated on fresh plates with one isolate per plate and incubated at the same temperature. After incubation, the diameter of the discoloration area (orange) was measured and bacterial isolates positive for siderophores were categorized as low (zone size 1–5 mm), moderate (zone size 6–10 mm) and high (zone size 11–15 mm), depending on the intensity of siderophore production. The experiment was repeated three times at different subculture intervals. All the bacterial isolates were maintained on CAS agar medium. HCN production by the bacterial isolates was detected by the method of Bakker and Schipper (1987). For HCN production, the bacterial isolates were grown on an HCN induction medium (30 g tryptic soy broth, 4.4 g glycine, 15 g agar $l^{-1}$) at 28°C for four days. For each bacterial isolate, 100 μl of inoculum (× $10^8$ cells/ml) was dropped in the centre of the plates. Then a disk of Whatman filter paper dipped in 0.5% picric acid and 2% $Na_2CO_3$ was placed in the lid of the Petri dish, which was then sealed with parafilm. After four days of incubation at 28°C, the orange-brown discoloration of the paper indicated HCN production. In the case of ammonia production, bacterial isolates were grown in peptone water (g/l: peptone 10 g, NaCl 5 g, pH 7) and incubated at 30°C for four days. One ml of Nessler reagent was put into each tube and the presence of yellow colour indicating ammonia production was recorded (Dye, 1962).

*Microbial inoculation and plant growth*

The bacterial strains *M. ciceri* RC3, *A. chroococcum* A4, and *Bacillus* PSB1 and PSB10, which were positive for IAA, siderophore, HCN and ammonia, were selected and used either alone or in combination to assess their effect on chickpea productivity. Seeds of chickpea (var. Avrodhi) were surface sterilized (Vincent, 1970) and soaked for two hours in three-day-old cultures using 10% gum arabic as adhesive to deliver approximately $10^8$ cells/ml of *Mesorhizobium*, $10^5$ cells/ml of *Azotobacter* and $10^8$ cells/ml each of *Bacillus* PSB1 and *Bacillus* PSB10. For combined inoculation, the liquid cultures were mixed in equal proportions. The inoculated seeds were sown in the experimental fields of the Faculty of Agricultural Sciences, A. M. U., Aligarh, during the winter season. The experimental soil was sandy clay loam and had organic carbon 0.4%, Kjeldahl N 0.75 g $kg^{-1}$, Olsen P 16 mg $kg^{-1}$, pH 7.4 and WHC 0.44 ml $g^{-1}$. The experiment was laid out in a randomized block design with 12 treatments, each replicated six times. The treatments were: $T_0$ control ($N_{20}P_{20}K_{20}$ kg/ha); $T_1$ *Mesorhizobium ciceri*; $T_2$ *Azotobacter chroococcum*; $T_3$ *Bacillus* PS1; $T_4$ *Bacillus* PSB10; $T_5$ *M. ciceri* + *Bacillus* PSB1; $T_6$ *M. ciceri* + *Bacillus* PSB10; $T_7$ *A. chroococcum* + *Bacillus* PSB1; $T_8$ *A. chroococcum* + *Bacillus* PSB10; $T_9$ *Bacillus* PSBI + *Bacillus* PSB10; $T_{10}$ *M. ciceri* + *A. chroococcum* + *Bacillus* PSB1; $T_{11}$ *M. ciceri* + *A. chroococcum* + *Bacillus* PSB10. The experiments were conducted for two consecutive years to ensure the reproducibility of the results. Plants were uprooted at 90 and 145 days after sowing (DAS) and were used to measure the total dry mass. Nodule number and nodule mass were recorded in ten plant samples uprooted randomly from each treatment, 90 and 145 days after sowing. The roots were carefully washed with tap water, and intact nodules were detached from the roots and counted. Nodules and plant samples were oven dried (80°C) and weighed. The N contents in roots and shoots were measured at 90 and 145 DAS by the method of Iswaran and Marwah (1980). The chlorophyll content in the foliage was estimated at 90 and 145 DAS (Arnon, 1949). Seed yield and grain protein (Lowry et al., 1951) were determined at harvest (145 DAS). The data of the two-year field trials were pooled and subjected to analysis of variance (ANOVA).

## Results and discussion

In the present study, strains of *Mesorhizobium* (N=10) were isolated from the nodules of chickpea plants, while *Azotobacter* (N=12) and phosphate-solubilizing bacteria (N=10) were isolated from the rhizospheres of mustard and tomato. Among the strains, *Mesorhizobium ciceri* RC3, *A. chroococcum* A4, *Bacillus* PSB1 and *Bacillus* PSB10 were found to be positive for ammonia, HCN, siderophore and IAA production (Table 1). Further, the bacterial cultures were screened for siderophore production on CAS agar medium. Among these tested cultures, *M. ciceri* RC3, *A. chroococcum* A4, *Bacillus* PSB1 and *Bacillus* PSB10 were found to be potential siderophore producers (zone size 11–15 mm), whereas the rest of the isolates were categorized as moderate siderophore producers (zone size 6–10 mm, data not shown). *Bacillus* PSB10 (zone size 15 mm) showed 36% greater siderophore production compared with *M. ciceri* RC3. The amount of IAA produced by *M. ciceri* RC3 and *A. chroococcum* A4 was 34.5 and 13.7 μg $ml^{-1}$, respectively, whereas the phosphate solubilizers, *Bacillus* PSBI and *Bacillus* PSB10, showed maximum IAA production of 19.3 and 17.4 μg $ml^{-1}$, respectively, in Luria Bertani broth supplemented with 100 μg $ml^{-1}$ of tryptophan. *M. ciceri* RC3 increased IAA production by 79, 98 and 152% over *Bacillus* PSB1, *Bacillus* PSB10 and *A. chroococcum* A4, respectively. Generally, the IAA production increased with increasing tryptophan

concentration for all the tested cultures. Among the PS bacteria, *Bacillus* PSB1 and *Bacillus* PSB10 produced a clear halo 4 and 5 mm in size, respectively, on solid Pikovskaya medium. In liquid culture medium, *Bacillus* PSB1 and *Bacillus* PSB10 solubilized a considerable amount of tricalcium phosphate (TCP) (7 and 8 μg $ml^{-1}$ by PSB1 and PSB10, respectively). The solubilization of TCP was coupled with a decrease in pH (from 7 to 5), which was the same for both PS strains. Other authors published similar evidence of siderophore (Frah et al., 2005) and IAA (Khan et al., 2002) production and of P solubilization by PSM (Kucey et al., 1989). The solubilization of insoluble P could possibly be due to the release of organic acids (Singal et al., 1994; Maliha et al., 2004). Bacterial cultures expressing high IAA, HCN and siderophore production and the solubilization of inorganic P were selected to assess their effect on chickpea crops under field conditions.

*Table 1*

Production of siderophore (1), HCN (2), ammonia (3) and the IAA and solubilization of tricalcium phosphate (TCP) on solid (4) and liquid (5) Pikovskaya medium by rhizospheric microorganisms

| Bacterial isolates | (1) | (2) | (3) | IAA(μg $ml^{-1}$) | | | TCP solubilized | | Change in pH |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 20 T* | 60 T* | 100 T* | (4) | (5) | |
| *Bacillus* sp. PSB1 | 13 | + | + | 11.3 | 15.7 | 19.3 | 4 | 7 | 5 |
| *Bacillus* sp. PSB10 | 15 | + | + | 8.0 | 12.9 | 17.4 | 5 | 8 | 5 |
| *M. ciceri* RC-3 | 11 | + | + | 23.5 | 27.9 | 34.5 | – | – | – |
| *A. chroococcum* A4 | 14 | + | + | 9.5 | 12.3 | 13.7 | – | – | – |

(1): chrome-azurol agar siderophore (mm); (4): zone size (mm); (5): μg $ml^{-1}$; T* = Tryptophan concentration (μg $ml^{-1}$); + = positive for ammonia and HCN; – = tested but negative for the measured parameters

The effect of $N_2$-fixing and PS bacteria, when used singly or in combination, on chickpea crops was variable (Tables 2 and 3). Single inoculation with *Mesorhizobium* significantly ($P \leq 0.05$) increased the total dry matter production in chickpea by 160 and 127% at 90 and 145 DAS, respectively, relative to the control treatment. In general, dual inoculation treatments significantly ($P \leq 0.05$) enhanced the dry matter production even further at the two stages of crop growth. The addition of *A. chroococcum* to the combinations *M. ciceri* + *Bacillus* PSB1 and *M. ciceri* + *Bacillus* PSB10 resulted in the maximum increase in total dry matter production, compared to single and dual inoculation treatments or the untreated control. Triple inoculation with *M. ciceri* + *A. chroococcum* + *Bacillus* PSB10 increased the dry matter by 71 and 9% at 90 and 145 DAS relative to single inoculation with *M. ciceri*, and showed a considerable increase over the other treatments. The symbiotic traits (e.g. nodule number, nodule mass) were enhanced dramatically at 90 and 145 DAS following dual inoculation with *M. ciceri* + PSB1 or *M. ciceri* + *Bacillus* PSB10 or triple inoculation with *M. ciceri* + *A. chroococcum* + PSB1 or *M. ciceri* + *A. chroococcum* + *Bacillus* PSB10, relative to the single inoculation with *M. ciceri*. However, nodulation declined considerably at 145 DAS compared to 90 DAS. The dry mass of nodules followed a trend similar to the number of nodules for

each treatment. Single inoculation with *M. ciceri* RC3, dual inoculation with *M. ciceri* + PSB1 or *M. ciceri* + *Bacillus* PSB10 or triple inoculation with *M. ciceri* + PSB1 + *Azotobacter* A4 or *M. ciceri* + *Bacillus* PSB10 + *Azotobacter* A4 significantly enhanced the seed yield (> 200%) relative to the control. Single inoculations or a combination of *Azotobacater* with either of the PS bacteria, however, showed a marginal increase in seed yield over the control. Among all the treatments, triple inoculation with *M. ciceri* + *Azotobacter* A4 + *Bacillus* PSB10 was found to be superior, enhancing the seed mass by 250% compared to the control. The highest chickpea grain protein content (309 mg plant$^{-1}$) was observed after triple inoculation (*M. ciceri* + *Azotobacter* A4 + *Bacillus* PSB10), followed by *M. ciceri* + *Azotobacter* A4 + *Bacillus* PSB1 (307 mg plant$^{-1}$). In general, dual inoculation treatments showed higher protein contents in chickpea seed compared to single inoculation treatments or the control.

A high chlorophyll content (> 0.67 mg plant$^{-1}$) was recorded in the foliage of chickpea plants inoculated with *M. ciceri* + *Bacillus* PSB1, *M. ciceri* + *Bacillus* PSB10 or the triple inoculation treatments, compared to single inoculation or the control treatment. Triple inoculation with *M. ciceri* + *A. chroococcum* + *Bacillus* PSB10 increased the chlorophyll content by 100 and 62% compared with dual inoculation with *Azotobacter* A4 + *Bacillus* PSB1 or *Azotobacter* A4 + *Bacillus* PSB10, respectively. The effect of microbial inoculation on the N contents in the roots and shoots of chickpea plants at 90 and 145 DAS varied significantly between treatments (Table 2). While no significant difference in root N content was observed following single or dual inoculation treatment at either 90 or 145 DAS, the N content in the shoots was greater at 145 DAS due to inoculation with *M. ciceri* + *Bacillus* PSB10 or *M. ciceri* + *A. chroococcum* + *Bacillus* PSB10, compared to 90 DAS.

*Table 2*
Co-inoculation effects of nitrogen-fixing and phosphate-solubilizing bacteria on growth, nodulation and seed yield of chickpea plants

| Treatments | Dry matter$^{+}$ | | Nodulation | | | | Seed yield$^{+}$ |
|---|---|---|---|---|---|---|---|
| | | | No. plant$^{-1}$ | | Dry mass$^{++}$ | | |
| | 90 DAS | 145 DAS | 90 DAS | 145 DAS | 90 DAS | 145 DAS | |
| *Mesorhizobium ciceri* (*M. c.*) RC3 | 12.8* | 24.6* | 45* | 41* | 290* | 260* | 12* |
| *Azotobacter chroococcum* (*A. c.*) A4 | 8.1* | 11.9 | 10 | 6 | 41 | 55 | 5 |
| *Bacillus* PSB1 | 6.7 | 11.1 | 15 | 9 | 33 | 89 | 5 |
| *Bacillus* PSB10 | 7.0* | 11.2 | 15 | 4 | 50 | 70 | 5 |
| *M. c.* RC3 + *Bacillus* PSB1 | 17.5* | 24.7* | 59* | 48* | 430* | 544* | 12* |
| *M. c.* RC3 + *Bacillus* PSB10 | 20.9* | 25.9* | 70* | 43* | 520* | 568* | 13* |
| *A. c.* A4 + *Bacillus* PSB1 | 11.5* | 14.2* | 14 | 7 | 120* | 42 | 6 |
| *A. c.* A4 + *Bacillus* PSB10 | 12.2* | 14.8* | 12 | 7 | 70* | 42 | 7* |
| *Bacillus* PSB 1+ *Bacillus* PSB10 | 9.2* | 12.4 | 12 | 8 | 60 | 80 | 7* |
| *M. c.* RC3 + *A. c.* A4 + *Bacillus* PSB1 | 21.3* | 26.4* | 72* | 49* | 435* | 572* | 13* |
| *M. c.* RC3 + *A. c.* A4 + *Bacillus* PSB10 | 21.9* | 26.9* | 75* | 47* | 522* | 575* | 14* |
| Control | 4.8 | 11.1 | 9 | 6 | 32 | 40 | 4 |
| LSD (P≤0.05) | 2.12 | 1.69 | 14.4 | 8.38 | 30.7 | 81.62 | 2.24 |

$^{+}$: g plant$^{-1}$; $^{++}$: mg plant$^{-1}$; Values are means of six replicates. * = Indicates values significantly different from the control at P≤0.05

*Table 3*
Co-inoculation effects of nitrogen-fixing and phosphate-solubilizing bacteria on seed protein, chlorophyll content, and N content in roots and shoots of chickpea plants

| Treatments | Seed protein (mg $g^{-1}$) | Chlorophyll (mg $g^{-1}$) | N content (mg $g^{-1}$) | | | |
|---|---|---|---|---|---|---|
| | | | Root | | Shoot | |
| | | | 90 DAS | 145 DAS | 90 DAS | 145 DAS |
| *Mesorhizobium ciceri* (*M. c.*) RC3 | 199.8* | 0.65* | 19* | 21* | 23* | 25* |
| *Azotobacter chroococcum* (*A. c.*) A4 | 190.8* | 0.32 | 16* | 18* | 21* | 23* |
| *Bacillus* PSB1 | 180.2 | 0.17 | 14 | 16* | 17 | 20* |
| *Bacillus* PSB10 | 185.4* | 0.24 | 16* | 18* | 20* | 21* |
| *M. c.* RC3 + *Bacillus* PSB1 | 273.6* | 0.67* | 21* | 23* | 25* | 27* |
| *M. c.* RC3 + *Bacillus* PSB10 | 306.0* | 0.67* | 21* | 23* | 25* | 28* |
| *A. c.* A4 + *Bacillus* PSB1 | 237.6* | 0.34* | 18* | 20* | 22* | 24* |
| *A. c.* A4 + *Bacillus* PSB10 | 262.8* | 0.42* | 19* | 23* | 23* | 25* |
| *Bacillus* PSB 1+ *Bacillus* PSB10 | 190* | 0.44* | 18* | 20* | 20* | 21* |
| *M. c.* RC3 + *A. c.* A4 + *Bacillus* PSB1 | 307.0* | 0.67* | 19* | 24* | 26* | 27* |
| *M. c.* RC3 + *A. c.* A4 + *Bacillus* PSB10 | 309.0* | 0.68* | 20* | 24* | 27* | 28* |
| Control | 176.4 | 0.17 | 13 | 14 | 17 | 17 |
| LSD (P≤0.05) | 7.47 | 0.16 | 2.13 | 1.52 | 1.89 | 1.95 |

Values are means of six replicates. * = Indicates values significantly different from the control at P≤0.05

The beneficial effects of $N_2$-fixing bacteria and PSM, used either alone or in combination, on legume crops were reported by Sahar et al. (2002) and Zaidi et al. (2003). The use of a symbiotic $N_2$-fixing bacterium (*Mesorhizobium*) or a symbiotic $N_2$ fixer (*Azotobacter*) along with PS bacteria stimulated the growth, nodulation and yield of chickpea plants. The tested bacterial cultures produced considerable amounts of plant growth-promoting substances, which not only promoted the growth of the chickpea plants but may also have played a significant role in the suppression of pathogens, leading eventually to increased chickpea productivity under field conditions. It is a well-accepted fact that P-solubilizing bacteria not only solubilize insoluble forms of P in the soil but also release considerable amounts of growth-promoting substances, e.g. auxin and giberellins (Sattar and Gaur, 1987). The availability of balanced nutrients (e.g. N by $N_2$ fixer, Fe by siderophore producers and P by PS bacteria) and the release of plant growth-regulating substances, as observed under *in vitro* conditions, might have resulted in the enhancement of the chickpea plants and consequently an increase in the seed yield. Further, the increased availability of phosphorus enhances the rhizobial activity in the rhizosphere, resulting in better nodulation (Saber et al., 2005), nitrogen fixation and finally seed yield. The beneficial effect of *Azotobacter* was not only due to its ability to release growth-promoting substances, but could also be attributed to the release of other metabolites (e.g. antibiotics) into the surrounding environment, thus inhibiting root pathogens (Kothari and Saraf, 1990). The present findings thus suggested that microbial

cultures should be established and proliferated under field conditions as a suitable microbial combination for raising the productivity of chickpea. In conclusion, it seems reasonable to suggest that the effects on chickpea plants that result from inoculation with these cultures can be attributed primarily to nutrient availability and secondarily to growth regulators. The present findings indicate that the joint application of $N_2$ fixers and PS bacteria was more effective than single inoculation treatments and could be an effective bioresource for developing cost-effective bioinoculants to improve chickpea productivity. Further, the application of $N_2$-fixing bacteria with organisms possessing plant growth-promoting activities could help to reduce external inputs under field conditions.

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# PERFORMANCE AND GENE EFFECTS FOR ROOT CHARACTERS AND MICRONUTRIENT UPTAKE IN WHEAT INOCULATED WITH ARBUSCULAR MYCORRHIZAL FUNGI AND *Azotobacter chroococcum*

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The present investigation was conducted to investigate the impact of bio-inoculants on the magnitude and direction of gene effects and mean performance for root length density, root biomass per plant, AMF colonization in roots and micronutrient uptake (Cu, Fe, Mn, Zn) in wheat under low input field conditions. The material for study comprised three wheat cultivars, WH 147 (low mineral input), WH 533 (drought-tolerant), Raj 3077 (high mineral input) and six generations ($P_1$, $P_2$, $F_1$, $F_2$, $BC_1$ and $BC_2$) of three crosses, namely WH 147 × WH 533, WH 533 × Raj 3077 and WH 147 × Raj 3077. The experiment was conducted in a randomized block design with three replications having three treatments, i.e. (i) control; (ii) inoculation with arbuscular mycorrhizal fungi (AMF, *Glomus fasciculatum*); (iii) dual inoculation with AMF and *Azotobacter chroococcum* (*Azc*). The fertilizer doses in all three treatments were 80 kg N + 40 kg P + 18 kg $ZnSO_4$ $ha^{-1}$. Root length density, root biomass per plant, AMF colonization in roots and Zn and Mn content were found to be maximum after dual inoculation with AMF+*Azc* in all three crosses. Joint scaling tests revealed that additive-dominance gene effects were mainly operative in governing the expression of root biomass, Cu and Zn content in all three crosses for all three treatments (i.e. control, AMF and AMF + *Azc*). Pedigree selection in crosses WH 147 × WH 533 and WH 147 × Raj 3077 could be effective for breeding pure lines of wheat for sustainable agriculture (low input genotypes responsive to biofertilizers such as AMF and *Azotobacter*).

**Key words:** wheat, *Azotobacter chroococcum*, arbuscular mycorrhizal fungi, gene effects, root length density, micronutrients

## Introduction

Root characteristics play an important role in the development of new wheat germplasm with improved drought tolerance, nutrient and water uptake efficiency, lodging resistance and tolerance to mineral toxicity. Concerted efforts are being made world-wide to develop nutrient use-efficient crop cultivars responsive to biofertilizers to increase crop yield and maintain good soil health

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(Manske et al., 1995). Inoculation with arbuscular mycorrhizal fungi (AMF) has been found to increase the availability of phosphorus and other nutrients in crop plants because of its symbiotic association with plant roots, colonizing cortical tissues and extending hyphae into the rhizosphere (Hetrick et al., 1996). The responsiveness of wheat varieties to microbes in terms of improved performance of various traits differs greatly and these differences are due to the genetic background of the varieties (Behl et al., 2003).

The present study was conducted to determine the effects of plant genotype and bioinoculation with AMF and AMF+*Azc* on the root traits and micronutrient uptake of three wheat crosses under low input conditions.

## Materials and methods

Three genetically diverse wheat genotypes suitable for diverse agro-ecological conditions namely WH 147 (low mineral input), WH 533 (water deficit) and Raj 3077 (high mineral input) were involved in three crosses. Six generations ($P_1$, $P_2$, $F_1$, $F_2$, $BC_1$ and $BC_2$) of the three crosses, namely WH 147 × WH 533, WH 533 × Raj 3077 and WH 147 × Raj 3077, were evaluated in a randomized block design with three replications under low input field conditions having three treatments, i.e. control, inoculation with arbuscular mycorrhizal fungi (AMF) and dual inoculation with AMF and *Azotobacter* (*Azc*). Two rows each of $F_1$s and parents, ten rows of $F_2$s and four rows of backcrosses were planted with row-to-row and plant-to-plant spacings of 30 and 10 cm, respectively.

For AMF inoculation, pearl millet (*Pennisetum glaucum*) roots infected with AMF *Glomus fasciculatum* were chopped into small pieces and mixed with soil in the furrows at the time of sowing. *Azotobacter chroococcum* mutant Mac 27 was grown on nitrogen-free Jensen medium (Jensen, 1951) containing 2% sucrose at 30°C for 72 h. For *Azc* inoculation wheat seeds were first treated with traditional jaggery or molasses solution prior to treatment with charcoal-based *Azc* (Mac 27) in a beaker and shaken thoroughly to facilitate uniform coating of the seeds with an inoculum containing $10^9$ CFU/ml. The number of colony-forming units (CFU) was determined by the plate count method (Narula et al., 2002). *Azotobacter*-treated seeds were kept in the shade for about an hour to dry before sowing, to ensure that the *Azc* inoculum adhered to the seeds. For dual inoculation wheat seeds pre-inoculated with *Azc* were co-inoculated with AMF.

Observations were made on five plants in the non-segregating generations (parents and $F_1$s), fifty plants in the $F_2$ and twenty-five plants in each of the backcross generations in each replication of all three crosses to record root length density, AMF colonization in roots, plant biomass per plant, and content and uptake of micronutrients, namely copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn). The mean and variances of individual families were calculated and used for the estimation of gene effects (Jinks and Jones, 1958). The adequacy of the additive-dominance model was determined by joint scaling tests (Cavalli, 1952).

Root biomass per plant (g): The roots of individual plants were weighed after washing and air drying on a top-pan electronic balance.

Root length density (cm): Root length density was measured according to the formula given by Tennant (1975):

Root length (R): Number of intercepts × length conversion factor

AMF colonization in roots (%): Mycorrhizal colonization in the roots was calculated by the modified Phillips and Hayman (1970) method.

Micronutrient (Cu, Fe, Mn and Zn) content in shoots (ppm): These were analysed using an atomic absorption spectrometer (AAS, HP PHILIPS, PU9200X). Maize starch (Reference Material 8432) from the National Institute of Standards and Technology, Gaithersburg, USA, was used as the standard.

## Results and discussion

*Mean performance*

In general, bio-inoculation with AMF or AMF + *Azc* was seen to have an impact on the mean performance of parents, $F_1$, $F_2$, $BC_1$ and $BC_2$ for all the traits. However, comparative evaluation of the magnitude of the impact for various traits in different crosses and their generations revealed that the effects were cross-specific. The estimation of mean performance (m) for root characters and micronutrients in three wheat crosses given different treatments is presented in Table 1. Root length density, root biomass per plant, AMF colonisation in roots, and Zn and Mn content exhibited maximum values for dual inoculation with AMF + *Azc* in all three crosses. The increases were significant after both AMF and AMF + *Azc* inoculation in the cross WH 533 × Raj 3077 for root length density, AMF infection in roots, root biomass per plant and Zn content, and in the crosses WH 147 × WH 533 and WH 147 × Raj 3077 for AMF infection in roots, root biomass per plant and Zn content, whereas the Mn content was significantly higher after dual inoculation with AMF + *Azc* in the crosses WH 533 × Raj 3077 and WH 147 × Raj 3077. Similar results were observed by Behl et al. (2003). After inoculation with AMF the Mn, Cu and Fe contents were lowest in the cross WH 147 × Raj 3077, whereas the Cu and Fe contents were highest in the cross WH 533 × Raj 3077.

*Table 1*
Mean performance (m) for root characters and micronutrients in three wheat crosses given different treatments

| Characters | Treatments | WH 147 × WH 533 | WH 533 × Raj 3077 | WH 147 × Raj 3077 | $C.D_{5\%}$ |
|---|---|---|---|---|---|
| Root length density (cm) | Control | 476.14±9.87 | 525.31±9.92 | 679.21±8.80 | |
| | AMF | 548.32±9.35 | 611.91±9.48* | 746.02±8.87 | 77.93 |
| | AMF+*Azc* | 642.92±8.98* | 735.50±11.53* | 764.35±7.70* | |
| AMF infection in roots (%) | Control | 19.07±2.06 | 19.80±2.44 | 19.09±2.31 | |
| | AMF | 34.91±2.90* | 37.17±2.81* | 30.63±3.09* | 3.58 |
| | AMF+*Azc* | 40.50±3.28* | 41.52±3.35* | 39.43±3.06* | |
| Root biomass per plant (g) | Control | 1.38±0.30 | 1.32±0.47 | 1.60±0.37 | |
| | AMF | 1.54±0.30* | 1.52±0.30* | 1.74±0.38* | 0.09 |
| | AMF+*Azc* | 1.77±0.34* | 1.75±0.35* | 1.88±0.31* | |
| Cu content (ppm) | Control | 6.11±1.29 | 10.14±0.92 | 8.65±1.18 | |
| | AMF | 8.00±1.18 | 11.27±1.26 | 8.50±1.26 | 2.14 |
| | AMF+*Azc* | 9.08±1.14* | 10.41±1.36 | 10.71±1.33 | |
| Fe content (ppm) | Control | 165.79±3.35 | 224.49±4.12 | 269.52±4.68 | |
| | AMF | 248.65±4.65 | 265.78±4.18 | 228.25±4.36 | 86.90 |
| | AMF+*Azc* | 266.75±4.11* | 248.57±4.41 | 236.92±4.50 | |
| Mn content (ppm) | Control | 45.18±2.70 | 44.80±2.06 | 52.06±2.15 | |
| | AMF | 49.73±2.17 | 51.09±2.45 | 49.96±2.08 | 6.38 |
| | AMF+*Azc* | 50.03±2.18 | 54.31±2.22* | 59.54±1.85* | |
| Zn content (ppm) | Control | 22.75±1.83 | 23.25±2.08 | 22.77±2.02 | |
| | AMF | 27.23±2.06* | 27.89±1.74* | 27.43±2.07* | 0.89 |
| | AMF+*Azc* | 29.62±1.70* | 29.12±1.38* | 29.89±1.88* | |

* = Significant at the 5% level

*Gene effects*

Non-significant chi-square values in the joint scaling tests (Cavalli, 1952) indicated the adequacy of the additive-dominance model, implying that the inheritance of root biomass, Cu and Zn content in all three crosses with all three treatments (i.e. control, AMF and AMF + *Azc*) was mainly governed by additive (d) and dominance (h) gene effects. The estimates of 'd' or 'h' were only significant for specific crosses/treatments, as presented in Table 2.

*Table 2*
Gene effects (additive and dominance) in three wheat crosses for root traits and micronutrients under different bio-inoculation treatments

| Characters | Treatments | WH 147 × WH 533 | | WH 533 × Raj 3077 | | WH 147 × Raj 3077 | |
|---|---|---|---|---|---|---|---|
| | | d | h | d | h | d | h |
| AMF infection | Control | digenic | digenic | digenic | digenic | 0.01±2.07 | –11.74±4.31** |
| in roots (%) | AMF | 3.43±2.81 | –11.57±5.46** | digenic | digenic | digenic | digenic |
| | AMF+*Azc* | digenic | digenic | 3.49±3.19 | –5.09±6.17 | –3.68±3.00 | –5.24±5.77 |
| Root biomass/ | Control | 0.01±0.30 | –0.16±0.62 | 0.11±0.46 | –0.02±0.83 | 0.14±0.37 | 0.39±0.73 |
| plant (g) | AMF | 0.06±0.30 | –0.07±0.60 | 0.07±0.31 | 0.05±0.50 | 0.03±0.39 | –0.22±0.69 |
| | AMF+*Azc* | 0.08±0.33 | 0.07±0.63 | –0.10±0.35 | 0.12±0.66 | –0.09±0.31 | 0.47±0.67 |
| Cu content | Control | –0.51±1.26 | 1.70±2.44 | 1.38±0.93 | 1.16±1.65 | 1.76±1.18 | 0.49±2.10 |
| (ppm) | AMF | –0.85±1.16 | 0.72±2.24 | 0.18±1.19 | –1.60±2.30 | 1.85±1.22 | –0.09±2.30 |
| | AMF+*Azc* | –0.09±1.12 | 1.86±2.13 | 0.74±1.31 | –1.52±2.59 | 0.74±1.29 | –0.58±2.31 |
| Fe content (ppm) | AMF+*Azc* | digenic | digenic | digenic | digenic | 17.00±4.42** | –9.79±8.13 |
| Mn content | Control | –4.42±2.76 | –3.93±4.20 | 2.10±2.08 | 8.59±3.59* | digenic | digenic |
| (ppm) | AMF | –2.17±2.14 | –5.17±4.26 | 4.06±2.40 | 4.93±4.50 | 2.81±2.08 | 3.01±3.49 |
| 5. | AMF+*Azc* | digenic | digenic | 3.00±2.18 | –0.65±3.95 | digenic | digenic |
| Zn content | Control | 0.72±1.79 | 0.75±3.23 | –0.04±2.11 | 0.74±3.38 | 0.68±1.99 | 0.31±3.57 |
| (ppm) | AMF | 1.08±1.99 | 0.87±3.70 | –0.45±1.75 | –0.14±2.59 | 0.56±2.03 | –0.15±3.68 |
| | AMF+*Azc* | 0.62±1.68 | 1.27±2.96 | 0.39±1.37 | 1.44±2.30 | 1.82±1.83 | –1.42±3.19 |

d = additive gene effects; h = dominance gene effects

Significant chi-square values in the joint scaling tests indicated the presence of digenic interactions (i.e. additive × additive, additive × dominance, dominance × dominance). These were exhibited for root length density in all three crosses and all three treatments, for Fe content in all three crosses in the control and AMF treatments, for AMF colonization in roots and Fe content in the crosses WH 147 × WH 533 and WH 533 × Raj 3077 in the control treatment, and for Mn content in the cross WH 147 × Raj 3077 in the control and AMF + *Azc* treatments (Table 3).

The root system of wheat has received comparatively less attention from plant biologists and plant breeders than the aboveground shoot. This is particularly true for field experiments. Although considerable efforts have been made to analyse genotypic variation for root traits in wheat, breeders have only rarely selected for root properties. The importance of AMF in regulating the water and nutrient uptake by plants under stress and improving their nutrient acquisition efficiency and yields has been documented (Manske and Vlek,

2002). However, only sporadic efforts have been made to study gene effects governing the response to bioinoculation and favourable plant–microbe interactions under low input systems. Such studies will be of utmost importance to decide the magnitude and direction of selection pressure in segregating generations for further improvement. In self-pollinated crops like wheat, dominance – being ephemeral in nature – cannot be exploited for tangible improvement. On the other hand, additive and additive × additive effects – being fixable – can be exploited through simple selection. In the present studies efforts were made to analyse genotypic variation in three wheat crosses for root traits governing to some extent the acquisition of micronutrients. Considerable additive and/or additive × additive gene effects were observed for root length density, root biomass per plant, AMF colonization in roots and micronutrient contents in plant samples. The studies revealed that pedigree selection in crosses WH 147 × WH 533 and WH 147 × Raj 3077 could be effective for breeding pure lines of wheat for sustainable agriculture (low input genotypes responsive to biofertilizers like AMF and *Azotobacter*).

*Table 3*

Estimation of digenic interactions in three wheat crosses for root traits and micronutrients under different bio-inoculation treatments

| Treatm. | Cross | d | h | i | j | l | E |
|---|---|---|---|---|---|---|---|
| 1. Root length density (cm) | | | | | | | |
| Control | WH 147 × WH533 | 25.73±3.38** | –297.20±13.98** | –192.00±8.85** | –51.47±7.39** | 115.47±11.01** | D |
| | WH 533 × Raj 3077 | –62.00±3.27** | 137.00±13.37** | 120.00±8.73** | 68.00±6.63** | 182.00±10.42** | C |
| | WH 147 × Raj 3077 | 1.34±3.10 | 1338.14±13.78** | 132.96±9.00** | –72.68±6.81** | –1012.48±10.69** | D |
| AMF | WH 147 × WH533 | 43.37±3.19** | 999.43±13.91** | 258.94±9.04** | –272.17±6.93** | –672.05±10.85** | D |
| | WH 533 × Raj 3077 | –35.08±3.09** | 1063.02±15.19** | 166.50±9.93** | 12.93±7.41 | –788.40±11.72** | D |
| | WH 147 × Raj 3077 | 29.38±3.24** | 931.21±14.61** | 128.26±9.45** | –83.95±7.36** | –7.55±11.32** | D |
| AMF+*Azo* | WH 147 × WH533 | 68.93±3.17** | 2138.51±14.33** | 566.28±9.27** | –902.11±7.20** | –1487.65±11.14** | D |
| | WH 533 × Raj 3077 | –69.02±3.53** | 2846.42±14.83** | 811.86±9.84** | 324.47±7.03** | –1917.55±11.37** | D |
| | WH 147 × Raj 3077 | –115.42±3.24** | –329.06±15.58** | –205.70±10.18** | 662.95±7.63** | 9.27±11.97 | D |
| 2. AMF infection in roots | | | | | | | |
| Control | WH 147 × WH533 | 5.31±1.51** | –44.88±5.54** | –3.76±3.47 | –6.87±3.00** | 40.99±4.43** | D |
| | WH 533 × Raj 3077 | 2.48±1.67 | 121.98±6.50** | 47.50±4.06** | –2.46±3.51 | –81.28±5.17** | D |
| AMF | WH 533 × Raj 3077 | 1.56±1.78 | 59.67±6.86** | 48.74±4.13** | –4.38±3.87 | –5.62±5.59 | - |
| | WH 147 × Raj 3077 | 3.75±1.85* | –42.37±6.76** | 1.00±4.37 | –8.50±3.48* | 48.74±5.33** | D |
| AMF+*Azo* | WH 147 × WH533 | 3.44±1.88 | –70.31±7.32** | –16.66±4.84** | –40.62±3.53** | 63.14±5.67** | D |
| 3. Fe content (ppm) | | | | | | | |
| Control | WH 147 × WH533 | –14.19±1.90** | 756.08±8.61** | 266.70±5.74** | 50.20±3.98** | –482.20±6.56** | D |
| | WH 533 × Raj 3077 | –17.38±2.21** | 429.88±9.16** | 129.02±5.97** | 78.79±4.57** | –306.95±7.08** | D |
| | WH 147 × Raj 3077 | 8.35±2.22** | –266.09±9.32** | –108.98±6.05** | 18.48±4.67** | 194.06±7.27** | D |
| AMF | WH 147 × WH533 | 11.12±2.17** | –338.29±9.50** | –131.52±6.16** | –72.44±4.75** | 242.02±7.42** | D |
| | WH 533 × Raj 3077 | 24.07±2.14** | –272.35±9.16** | –80.14±5.98** | –46.00±4.52** | 189.74±7.09** | D |
| | WH 147 × Raj 3077 | 18.82±2.19** | –363.63±9.37** | –207.76±6.07** | 60.60±4.71** | 166.82±7.30** | D |
| AMF+*Azo* | WH 147 × WH533 | –3.48±2.19 | 39.60±8.39* | 83.94±5.45** | 34.51±4.26** | 53.69±6.55** | C |
| | WH 533 × Raj 3077 | –3.53±2.23 | 105.53±9.04** | 41.16±5.88** | 85.14±4.54** | –49.90±7.03** | D |
| 4. Mn content (ppm) | | | | | | | |
| Control | WH 147 × Raj 3077 | –1.46±1.57 | 73.29±6.16** | 21.14±4.09** | 1.27±2.93 | –46.69±4.72** | D |
| AMF+*Azo* | WH 147 × WH533 | 0.78±1.61 | –29.68±6.60 | –26.44±4.24** | 0.24±3.40 | –0.48±5.16 | – |
| | WH 147 × Raj 3077 | 9.32±1.44** | 27.74±6.65** | –2.46±4.45 | –19.38±3.03** | –17.12±5.03** | D |

d = additive gene effects, h = dominance gene effects, i = additive × additive interactions, j = additive × dominance interactions, l = dominance × dominance interactions, E = epistasis, D = duplicate epistasis, C = complementary epistasis

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# ASSESSMENT OF GENOTYPE × ENVIRONMENT INTERACTIONS FOR YIELD AND MORPHINE CONTENT IN OPIUM POPPY (*Papaver somniferum* L.)

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The analysis of the genotype × environmental interaction, which indicates the stability of genotypes, has always been part of plant breeding programmes before the release of varieties for commercial cultivation. In the present investigation the stability of seed yield, opium yield and morphine content in 11 advanced breeding lines of opium poppy was evaluated over five years. Combined ANOVA showed that both the main effects and interactions were significant, indicating the presence of genotype × environment interactions. The average seed yield and opium yield ranged from 10.41–16.92 q/ha and 45.21–59.85 kg/ha, respectively. Stability analysis involving the parameters bi, $S^2di$, $\lambda i$, $s^2$, $\delta^2$, Wi, $r^2$ and CV identified four genotypes (BR330, BR333, BR229 and BR243) as ideal and stable for the traits studied. The level of association among different parameters obtained using Spearman's rank correlation showed that Eberhart and Russell's deviation from regression ($S^2di$) was significantly and positively associated with $s^2$, $\lambda i$, $\delta^2 i$ and CV and negatively with $r^2$. The parameters $\lambda i$, $s^2$, $\delta^2 i$ and CV also showed positive mutual association.

**Key words:** *Papaver somniferum*, stability, coefficient of determination, regression, linear response

## Introduction

The interaction between genotype and environment is an important consideration for plant breeders when screening for stable and promising genotypes. The genotype × environment (G × E) interaction is associated with the differential performance of materials tested at different locations/years, and its influence on the selection and prediction of adaptable genotypes for commercial cultivation (Lin et al., 1986; Annicchiarico, 1997). The effects that the genotypes and environment exert on the genotype × environment interaction (G × E) are statistically non-additive, which indicates that differences in yields between genotypes depend on the environment (Yue et al., 1997). It helps to

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determine an optimum breeding strategy for breeding for specific or wide adaptation, which depends on the expression of stability (Yue et al., 1997; Tesemma et al., 1998). Stability can be defined as non-erratic performance (minimal or low interactions) with respect to agronomic traits (Allard and Bradshaw, 1964). It also denotes consistency in rank relative to other genotypes in a given set of environments (Yue et al., 1997). Yield stability is the ability of a genotype to avoid substantial fluctuations in yield over a range of environments. Differences in yield over environments (G × E) hinder the gain from selection as it reduces the association between genotypic and phenotypic values and makes it difficult to select genotypes that are widely adapted and stable.

Among the various parametric models based on simple linear regression analysis, the model proposed by Eberhart and Russell (1966) is widely used as it interprets the variance of the regression deviation ($S^2di$) as a measure of genotype stability and the linear regression coefficient (bi) as a measure of genotype adaptability. Eberhart and Russell (1966) describe a stable variety as one where the regression coefficient bi=1 and the minimum deviation from regression $S^2di=0$. The other commonly used parameters are: $\alpha i$ (linear response of genotypes to the environmental effect) and $\lambda i$ (deviation from linear response), proposed by Tai (1971), which can be regarded as a special form of the regression parameters bi and $S^2di$, when the environment index is assumed to be random (Lin et al., 1986). Wi (ecovalence), given by Wricke (1962), is the contribution of each genotype to the interaction sum of squares, while $\delta^2i$ (stability variance) is a modified form of Wi, which makes it possible to give an unbiased estimate of G × E variance (stability variance) for each genotype (Shukla, 1972). The coefficient of variation (CV) was used by Francis and Kannenberg (1978) as a conventional stability measure, while the coefficient of determination ($r^2$) and the environmental variance ($s^2$) (Becker and Léon, 1988; Nissila, 1992) are also commonly used as stability parameters.

The opium poppy (*Papaver somniferum* L.) is grown in India primarily for opium latex, which contains a diverse range of pharmaceutically important alkaloids. Besides latex, poppy seeds, as a by-product, are highly nutritive and are used in the preparation of breads, curries, sweets and confectionaries (Singh et al., 1995). India is one of the world's largest producers and exporters of opium poppy products. Hence, to meet the domestic and international demand for opium products, there is always a call to develop new, high-yielding varieties. The newly developed varieties must be tested for their adaptability and stability in different environments prior to their release for commercial cultivation. Several studies on stability parameters have been done on opium poppy (Singh and Shukla, 2001; Singh et al., 2005), but these studies deal with different materials, locations and environments. Hence, the present investigation aimed (i) to select promising, stable genotypes from advanced breeding material of opium poppy following the Eberhart and Russell (1966) model, and (ii) to compare various statistical models in order to test the suitability and authenticity of the Eberhart and Russell (1966) model.

## Materials and methods

The material, comprising 11 advanced, pure breeding lines (BR330, BR336, BR333, BR335, BR328, BR229, BR238, BR307, BR313, BR291 and BR243) of opium poppy obtained through various breeding programmes, was evaluated in a randomized block design (RBD) with three replications during the crop years 1999–2000 to 2003–2004. The trials were carried out in the experimental fields of the National Botanical Research Institute, Lucknow (26°40' N, 80°45' E, 129 m above sea level). The average rainfall during the crop period (November – April) ranged from 3.8 mm to 21.9 mm and the average day and night temperatures varied between 22–38.0°C and 4.1–20.5°C, respectively. The plot size was 4.8 $m^2$ (having eight rows 25 cm apart). The plants were spaced 10 cm apart within rows. Standard cultural practices were followed throughout the crop season. The middle six rows of each plot were used as the harvest area for opium latex and seed yield. Opium latex was collected from fully grown green capsules and subsequently seeds were collected from the dried matured capsules. The morphine content of the opium samples was estimated through the colorimetric method (Pride and Stern, 1954).

The mean values of each trait were subjected to combined analysis of variance followed by stability analysis using various parametric methods for testing phenotypic stability, as proposed by Eberhart and Russell (1966), Tai (1971), Wricke (1962), Shukla (1972), Francis and Kannenberg (1978), Becker and Léon (1988) and Nissila (1992). The software Windowstat (Indostat Services, Hyderabad, India) was used for the statistical analysis of the data.

## Results and discussion

The opium poppy is a restricted crop and is grown by only licensed countries under high surveillance of INCB (International Narcotics Control Board), Vienna. In India only three states (U.P., M.P. and Rajasthan) are presently engaged in licit poppy cultivation under the strict vigilance of the Narcotics Department of India. The present investigation was carried out at Lucknow, the capital of U.P., over five years to record the data for stability analysis. Earlier Yue et al. (1997) suggested that data obtained from multiple years are more reliable for stability. The genotypes tested showed inconsistent performance for all the traits, which was reflected in their variable ranks over different environments. Averaged over years, the genotype BR330 gave the highest seed yield (16.92 q/ha), followed by BR328 (16.43 q/ha) and BR333 (16.20 q/ha) (Table 1), while genotype BR330 exhibited maximum opium yield (59.85 kg/ha), followed by BR243 (58.91 kg/ha) and BR 238 (57.89 kg/ha) (Table 2). The morphine content was highest in BR328 (17.96%) (Table 3).

Combined analysis of variance on all three traits showed that all the main as well as interaction effects were statistically significant (Table 4), which indicated that the presence of genotype and environment differences governed the expression of these traits. The significant variances due to G × E also confirmed the above findings. The partitioning of the interaction effects showed that the environment (linear) component was highly significant for all the traits, while the linear component of the environmental interaction {G × E (linear)} was significant for opium and seed yield. The significant pooled deviation (non-linear) suggests that the performance of different genotypes fluctuated

considerably in respect to their stability for respective characters. The significance of environmental effects and their interactions warrants further analysis of the stability and adaptation of the genotypes. Eberhart and Russell (1966) proposed that an ideal population of genotypes is one which has high yield over a broad range of environments, a value of the regression coefficient (bi) equal to 1 and a value of the deviation mean square equal to zero. The mean (gi) and non-linear component ($S^2di$) were considered for the stability test and the regression coefficient (bi) was used for testing the varietal response.

*Table 1*

Mean value (q/ha) and stability parameters of seed yield in opium poppy genotypes, evaluated over five years

| Genotypes | Mean± SE | bi | $S^2di$ | $r^2$ | $s^2$ | λi | $\delta^2i$ | Wi | CV |
|---|---|---|---|---|---|---|---|---|---|
| BR330 | 16.92±0.38 | 0.95 | 0.31 | 0.87 | 2.36 | 3.71 | –0.12 | 1.23 | 8.70 |
| BR336 | 11.68±0.86 | 1.37 | 11.15** | 0.34 | 12.74 | 93.86 | 10.18** | 34.99 | 28.41 |
| BR333 | 16.20±0.39 | 0.90 | 0.83 | 0.72 | 2.53 | 8.63 | 0.36* | 2.87 | 9.22 |
| BR335 | 13.47±0.78 | 0.74 | 12.23** | 0.12 | 10.51 | 118.8 | 10.96** | 37.56 | 22.48 |
| BR328 | 16.43±0.35 | 0.95 | –0.08 | 0.98 | 2.07 | 0.08 | –0.50** | 0.05 | 8.22 |
| BR229 | 15.67±0.43 | 0.82 | 1.96** | 0.49 | 3.06 | 19.44 | 1.45** | 6.45 | 10.60 |
| BR238 | 13.29±0.62 | 1.45 | 1.83** | 0.77 | 6.27 | 15.76 | 1.82** | 7.66 | 18.11 |
| BR307 | 12.32±0.92 | 2.31 | 2.83** | 0.85 | 14.36 | 18.79 | 6.94** | 24.41 | 28.85 |
| BR313 | 10.41±0.74 | 1.26 | 7.40** | 0.39 | 9.26 | 64.30 | 6.55** | 23.12 | 27.41 |
| BR291 | 13.06±0.76 | –0.7 | 11.25** | 0.12 | 9.63 | 143.01 | 17.96** | 60.48 | 22.51 |
| BR243 | 15.14±0.39 | 0.94 | 0.48 | 0.82 | 2.45 | 5.30 | 0.02 | 1.75 | 9.94 |

*,** significant at the 5% and 1% level, respectively

*Table 2*

Mean value (kg/ha) and stability parameters of opium yield in opium poppy genotypes, evaluated over five years

| Genotypes | Mean± SE | bi | $S^2di$ | $r^2$ | $s^2$ | λi | $\delta^2i$ | Wi | CV |
|---|---|---|---|---|---|---|---|---|---|
| BR330 | 59.85±1.35 | 0.93 | 11.50** | 0.63 | 25.28 | 12.08 | 10.16** | 37.45 | 8.70 |
| BR336 | 47.12±1.06 | –0.22 | 21.52** | 0.05 | 17.70 | 23.96 | 53.35** | 178.74 | 8.70 |
| BR333 | 57.03±1.09 | 0.94 | 4.69** | 0.80 | 20.75 | 5.41 | 3.87** | 16.85 | 7.42 |
| BR335 | 54.79±2.00 | 1.79 | 8.55** | 0.89 | 67.26 | 8.54 | 21.78** | 75.48 | 14.15 |
| BR328 | 57.46±1.22 | 0.85 | 12.47** | 0.57 | 23.47 | 13.11 | 11.44** | 41.65 | 8.20 |
| BR229 | 55.88±1.03 | 0.86 | 4.02** | 0.79 | 17.46 | 4.79 | 3.63** | 16.09 | 7.12 |
| BR238 | 57.89±1.25 | 1.14 | 2.42* | 0.91 | 26.93 | 3.14 | 2.19** | 11.37 | 8.35 |
| BR307 | 51.28±1.65 | 1.39 | 11.22** | 0.80 | 45.35 | 11.35 | 13.32** | 47.80 | 12.48 |
| BR313 | 45.21±1.40 | 0.73 | 28.68** | 0.31 | 32.31 | 29.43 | 27.46** | 94.06 | 11.94 |
| BR291 | 53.13±1.76 | 1.61 | 2.68** | 0.95 | 50.82 | 3.26 | 10.34** | 38.03 | 12.82 |
| BR243 | 58.91±1.22 | 0.97 | 9.64** | 0.69 | 25.40 | 10.23 | 8.36** | 31.54 | 8.05 |

*,** significant at the 5% and 1% level, respectively

*Table 3*
Mean value (%) and stability parameters of morphine content in opium poppy genotypes, evaluated over five years

| Genotypes | Mean± SE | bi | $S^2di$ | $r^2$ | $s^2$ | λi | $\delta^2i$ | Wi | CV |
|---|---|---|---|---|---|---|---|---|---|
| BR330 | 16.77±0.77 | 0.67 | 0.19 | 0.52 | 0.50 | 2.19 | 0.29* | 1.22 | 4.04 |
| BR336 | 15.50±0.26 | 1.21 | 0.10 | 0.83 | 1.03 | 1.45 | 0.17 | 0.79 | 6.53 |
| BR333 | 15.89±0.15 | 0.66 | –0.05 | 0.81 | 0.31 | 0.54 | 0.08 | 0.51 | 3.60 |
| BR335 | 16.22±0.38 | 1.44 | 1.11** | 0.57 | 2.15 | 7.51 | 1.20** | 4.18 | 9.11 |
| BR328 | 17.96±0.24 | 0.78 | 0.27 | 0.54 | 0.66 | 2.70 | 0.33** | 1.32 | 5.10 |
| BR229 | 15.75±0.24 | 0.9 | 0.21 | 0.65 | 0.73 | 2.25 | 0.24 | 1.05 | 5.92 |
| BR238 | 14.78±0.25 | 1.17 | 0.08 | 0.84 | 0.95 | 1.29 | 0.13 | 0.68 | 6.50 |
| BR307 | 15.85±0.38 | 1.45 | 0.65** | 0.68 | 1.82 | 4.68 | 0.78** | 2.80 | 9.25 |
| BR313 | 16.45±0.33 | 1.48 | 0.29 | 0.80 | 1.59 | 2.50 | 0.47** | 1.78 | 7.71 |
| BR291 | 16.35±0.34 | 0.25 | 1.45** | 0.03 | 1.22 | 11.46 | 1.78** | 6.06 | 7.98 |
| BR243 | 14.72±0.37 | 1.01 | 1.84** | 0.29 | 2.07 | 12.76 | 1.73** | 5.90 | 9.77 |

*, ** significant at the 5% and 1% level, respectively

*Table 4*
Mean squares of the combined analysis of variance for seed yield, opium yield and morphine content in opium poppy evaluated over five years

| Source | df | Mean square | | |
|---|---|---|---|---|
| | | Seed yield | Opium yield | Morphine content |
| Genotypes | 10 | 23.06** | 114.91** | 4.13** |
| Environments | 4 | 25.11** | 205.47** | 6.46** |
| Env. + (G × E) | 44 | 6.84** | 32.07** | 1.18** |
| G × E | 40 | 5.01** | 14.73** | 0.66 |
| E (linear) | 1 | 100.43** | 821.86** | 25.83** |
| G × E (L) | 10 | 4.69** | 20.86** | 0.36 |
| Pooled deviation | 33 | 4.65** | 11.53** | 0.69* |
| Pooled error | 100 | 0.08 | 0.87 | 0.12 |

*, ** significant at the 5% and 1% level, respectively

The value of $S^2di$ was significant for all but four of the genotypes, suggesting that all the genotypes gave a highly sensitive, unpredictable response to environmental changes for seed yield. Genotypes BR330, BR328, BR243 and BR333 exhibited high mean yield, a non-significant deviation from regression ($S^2di$) and a regression coefficient near to unity (bi=1), which suggests that these genotypes might be considered as stable over environments (Table 1). Besides this, genotypes BR335 and BR291 had low bi values with lower yield. These genotypes may be adapted to poor environments. Genotypes BR336, BR238 and BR307 had regression coefficients significantly greater than 1, which indicates the sensitive response of these genotypes to changes in environmental conditions and their specific adaptation to favourable conditions. So despite their advantages these genotypes could fail miserably in a poor environment. Other models consider genotypes exhibiting low environmental variance ($s^2$), low coefficient of variation (CV) (Lin et al., 1986), low stability variance ($\delta^2i$) and

deviation from linear response approaching unity ($\lambda i=1$) (Flores et al., 1998) to be stable. On this basis the low coefficient of variation, stability variance and environmental variance of genotypes BR328, BR330 BR333 and BR243 confirmed the stability observed using the Eberhart and Russell (1966) model. In general, it was observed that genotypes with high bi values tended to have higher environmental variance and coefficient of variation and vice versa. The value of $r^2$ was high for all but two of the genotypes, indicating environmental effects as the main determinant of phenotypic performance (Table 1). A lower value of ecovalence (Wi) is an indication of genotypic stability (Wricke, 1962), showing genotypes BR328, BR330 and BR243 to be stable.

The performance of opium poppy is highly erratic, depending on environmental changes, especially for opium yield (Singh et al., 1995; 2005). This can also be demonstrated from the present investigation, where all the genotypes showed highly significant non-linear components ($S^2di$), indicating unpredictability and high sensitivity to environmental changes. Three genotypes, BR243, BR333 and BR229, can be identified as stable, since these had high mean yield values, bi values approaching unity and low deviation from regression ($S^2di$) (Table 2). The lower values of CV and $s^2$ for these genotypes also confirmed their stability. Genotypes BR336 and BR313 had the lowest mean yield and bi values less than one, indicating better adaptation to poor environments, while the genotypes BR335, BR238 and BR291 were found to be more suitable for favourable environments, as indicated by bi values >1.

Genotypes BR229 and BR238 were found to be most stable for morphine content, since they had bi values near to 1, low non-significant $S^2di$, minimum CV and environmental variances, and other stability parameters indicative of stability (Table 3). Genotypes BR336, BR335, BR238, BR307 and BR313 had b value >1, which indicated their responsiveness to variable environmental conditions and their specific adaptation to favourable ones. Regression coefficient values <1 were recorded for BR330, BR333 and BR291, indicating better adaptation to poor environments. Lower values of CV and environmental variance were recorded for BR333, BR330 and BR328. BR333 showed the lowest values of Wi and $\delta^2 i$, while BR238 and BR336 gave the highest values of $r^2$.

The correlations between mean yield and stability parameters were also calculated to determine the interrelationship of each stability parameter with yield and among themselves (Table 5). The nature of the correlation between yield and stability parameters can provide guidelines for the selection of high-yielding, stable genotypes. It was interesting to note that mean yield performance and stability parameters had low or negative association, but significant positive correlations were observed between most of the stability parameters, with the exception of $r^2$. This suggested that any of the parameters could be used to categorize stable genotypes except $r^2$, which showed a negative association with the other parameters. Similar results were reported by Mekib (2003) and Tesemma et al. (1998). The regression parameters bi and $r^2$ showed a low negative correlation with $S^2di$ and $\lambda i$, suggesting that bi and $r^2$ do not measure the same aspects of stability, so caution is required during stability

analysis using these parameters. The association between the bi, $S^2di$ and CV values was highly significant, indicating that genotypes with high values for these parameters were unstable. Becker (1981) reported consistently strong correlations between environmental variance and regression coefficient on the one hand and between ecovalance and deviation mean square on the other. He also noted that such correlations are to be expected, since these stability parameters have a biometrical relationship. Wricke's (1962) ecovalance was not included in the correlation study, since the correlation values of ecovalance and stability variance (Shukla, 1972) were found to be similar due to the same ranking. The deviation from regression $S^2di$ and $\lambda i$ showed a tight correlation, proving that $\lambda i$ (deviation from linear response) is equivalent to the $S^2di$ of Eberhart and Russell (1966). Similar findings were reported by Lin et al. (1986) and Flores et al. (1998).

It was concluded from the present investigation that the Eberhart and Russell (1966) model can be reliably used for identifying stable genotypes over environments, as it uses both yield and stability parameters to exploit the useful effect of the G × E interaction and make selection more precise. Based on the present study, four genotypes, BR330, BR333, BR229 and BR243, were identified as ideal and stable and are now being tested in demonstration plots aimed at the release of commercial cultivars suited to the local conditions.

*Table 5*

Spearman's rank correlation coefficients (rs) between the stability parameters for seed yield, opium yield and morphine content

| | | bi | $S^2di$ | $r^2$ | $S^2$ | $\lambda i$ | $\delta^2 i$ | CV |
|---|---|---|---|---|---|---|---|---|
| Yield | SY | –0.36 | –0.74* | 0.56 | –0.83** | –0.71* | –0.76* | –0.94** |
| | OY | –0.15 | –0.33 | 0.14 | –0.23 | –0.33 | –0.71 | –0.50 |
| | MC | –0.24 | 0.05 | –0.36 | –0.30 | 0.07 | 0.17 | –0.38 |
| bi | SY | | –0.21 | 0.47 | 0.21 | –0.30 | 0.97** | 0.81** |
| | OY | | –0.66 | 0.89** | 0.74* | –0.66* | –0.24 | 0.45 |
| | MC | | 0.17 | 0.45 | 0.64* | 0.11 | 0.11 | 0.54 |
| $S^2di$ | SY | | | –0.89** | 0.87** | 0.98** | 0.97** | 0.81** |
| | OY | | | –0.89 | 0.14 | 0.99** | 0.75** | 0.15 |
| | MC | | | –0.73* | 0.78* | 0.99* | 0.98** | 0.82** |
| $r^2$ | SY | | | | –0.61 | –0.93** | –0.83** | –0.55 |
| | OY | | | | 0.54 | –0.89** | –0.47 | –0.25 |
| | MC | | | | –0.19 | –0.76** | –0.76** | –0.26 |
| $s^2$ | SY | | | | | 0.81** | 0.91** | 0.95** |
| | OY | | | | | –0.14 | 0.29 | 0.83** |
| | MC | | | | | 0.74 | 0.73* | 0.96** |
| $\lambda i$ | SY | | | | | | 0.95** | 0.76** |
| | OY | | | | | | 0.75** | 0.97** |
| | MC | | | | | | 0.15 | 0.78** |
| $\delta^2 i$ | SY | | | | | | | 0.84** |
| | OY | | | | | | | 0.66* |
| | MC | | | | | | | 0.77** |

SY = Seed yield, OY = Opium yield, MC = Morphine content; *, ** significant at the 5% and 1% level, respectively

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# CORRELATIONS AND PATH ANALYSIS OF YIELD COMPONENTS IN SYNTHETIC VARIETIES OF SUNFLOWER (*Helianthus annuus* L.)

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This study was made in order to determine the correlations between seed yield and some yield components, and the direct and indirect effects of these characters on seed yield in sunflower. Four experimental synthetic varieties (Syn 1s), their parental mixtures (Syn Os) and two standard varieties (open pollinated: Vniimk 8931, and commercial hybrid: Sunbred-281) were evaluated in replicated field trials under Turkish conditions in 1995, 1996 and 1997. Agronomic characteristics such as plant height, head diameter, number of seeds per head, 1000-seed weight and seed yield were observed for correlations and path coefficient analysis.

According to the results, seed yield gave significant positive correlations with plant height, head diameter, number of seeds per head and 1000-seed weight. The highest positive correlation was observed between seed yield and number of seeds per head (r= 0.890**). Path analysis indicated that the number of seeds per head gave the greatest direct effect (+0.7269) on seed yield, followed by 1000-seed weight (+0.3215) and head diameter (+0.1689). The percentage of direct effects on seed yield was 80.8%, 50.6% and 24.0% for number of seeds per head, 1000-seed weight and head diameter, respectively.

**Key words:** sunflower, synthetic variety, yield components, correlation, path analysis

## Introduction

Sunflower (*Helianthus annuus* L.) is the most important oil crop in Turkey. It is grown on about 550,000 ha with an annual seed production of 800,000 tons. Demand for sunflower as both oilseed and unrefined oil has increased recently. Sunflower has been imported to satisfy the increasing demand for oilseed and unrefined oil for a long time.

Sunflower seed production should be increased in order to reduce imports. Production can be increased by using modern cultural practices and breeding programmes. Higher yields depend on successful results in plant breeding

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studies. A knowledge of the relationship between yield and agronomic traits is important in plant breeding, especially for selection. These relationships can be measured by the correlation coefficient. If the aim of a planned breeding programme is to increase seed yield, a knowledge of the correlations between various agronomic characters and the yield provides important guidelines for breeding programmes. Many earlier studies indicated that agronomic characters such as plant height, head diameter, number of seeds per head, 1000-seed weight, seed weight and seed weight per head were positively and significantly associated with seed yield in sunflower (Alvarez et al., 1992; Marinkoviç, 1992; Punia and Gill, 1994; Narayana and Patel, 1998).

The study was carried out to determine the direct and indirect effects of certain yield components on seed yield and to measure correlations between seed yield and its components in synthetic sunflower varieties.

## Materials and methods

In the study twelve inbred sunflower lines originating from two distinct sources were used to create experimental synthetic varieties. The inbred lines, which were selfed for seven generations, were chosen for their phenotypical superiority and simultaneous flowering times. The twelve inbred lines were put into three groups and three experimental synthetics were formed, each composed of four lines. Additionally all twelve inbred lines were also used to create another synthetic variety.

The parental lines were first crossed artifically by hand in 1994 and this was repeated in the following two years to obtain Syn 1 seeds for the experiments. Additionally four Syn 0s were obtained by mixing the seeds of the parental lines in equal amounts. Two commercial cultivars, an open-pollinated cultivar, Vniimk 8931 and a hybrid cultivar, Sunbred-281, were also included in the study. Field experiments including ten genotypes (four Syn 1s, four Syn 0s and two commercial cultivars) were conducted in a randomized block design with four replications in 1995, 1996 and 1997. The experiments were carried out at the Research and Experimental Farm, Faculty of Agriculture, Uludağ University, Bursa, Turkey. Row spacing was 0.70 m and the plot area was 19.6 $m^2$ at harvest. Bursa is located in the Marmara Region, with an average 700 mm annual rainfall. Insufficient rainfall during summer greatly limits the yield in this area.

In the study, five agronomic characters, namely plant height (cm), head diameter (cm), number of seeds per head, 1000-seed weight (g) and seed yield (kg/ha) were observed in each of the three-year experiments. Twenty plants were randomly selected for these observations. Simple correlations were calculated between seed yield and yield components for individual years and over the mean of three years. The significance of the correlation coefficients was tested at the 5% and 1% probability levels. Additionally, in order to determine the direct and indirect effects of the other characters on the seed yield, path analysis was carried out as suggested by Li (1968).

## Results and discussion

Correlation coefficients between the seed yield and yield components in a genotypical population including synthetic varieties, a mixture of the parental lines and commercial cultivars are given in Table 1. According to the three-year results of the research, seed yield was found to be in positive significant

*Table 1*
Correlation coefficients among five characters for synthetic varieties of sunflower[(1)]

| Character | Plant height | Head diameter | No. of seeds/head | 1000-seed weight |
|---|---|---|---|---|
| Seed yield | 0.363* | 0.478** | 0.657** | 0.466** |
| | 0.522** | 0.683** | 0.938** | 0.661** |
| | 0.457** | 0.182 | 0.794** | 0.545** |
| | 0.589** | 0.696** | 0.890** | 0.631** |
| Plant height | – | 0.039 | 0.230 | 0.008 |
| | | 0.151 | 0.331* | 0.660** |
| | | 0.420** | 0.593** | 0.195 |
| | | 0.349* | 0.620** | 0.266 |
| Head diameter | – | – | 0.026 | 0.536** |
| | | | 0.657** | 0.406** |
| | | | 0.237 | 0.117 |
| | | | 0.451** | 0.626** |
| No.of seeds/head | – | – | – | –0.240 |
| | | | | 0.374* |
| | | | | 0.292 |
| | | | | 0.283 |

*, ** Significant at P = 0.05 and P = 0.01, respectively; [(1)] Correlation coefficients are values in 1995, 1996, 1997 and mean of 3 years, downward, respectively

association with the plant height (r=+0.589) (Fig. 1), head diameter (r=+0.696) (Fig. 2), number of seeds per head (r=+0.890) (Fig. 3) and 1000-seed weight (r=+0.631) (Fig. 4). Seed yield was positively and significantly associated with all the characters studied except head diameter in 1997. On the other hand, positive significant associations between seed yield and the number of seeds per head gave the highest correlation coefficients. These values were +0.657, +0.938, +0.794 and +0.890 for 1995, 1996, 1997 and the 3-year mean, respectively (Table 1 and Fig. 3). These results are in agreement with those reported by Patil et al. (1996), who found that the highest correlations were observed between seed yield and number of seeds per head. In correlation and path analysis on 45 sunflower genotypes, Gill et al. (1997) found that there were positive significant associations between seed yield per plant and plant height, stem diameter, head diameter, 1000-seed weight and number of seeds per head. Similar results were reported in earlier studies (Vanisree et al., 1988; Marinkoviç, 1992; Kıllı and Gencer, 1992; Punia and Gill, 1994; Doddamani et al., 1997).

Averaged over the three years, plant height was positively and significantly associated with head diameter (Fig. 5) and number of seeds per head (Fig. 6), with correlation coefficients of +0.349 and +0.620, respectively. The association between plant height and 1000-seed weight was positive, but not significant. There were positive associations between head diameter and both number of seeds per head (r=+0.451) (Fig. 7) and 1000-seed weight (r=+0.626) (Fig. 8). The correlation between number of seeds per head and 1000-seed weight was positive but not significant (r=+0.283). Generally a negative correlation is expected between these two characters. In the present case, although the correlation was positive, it was very weak.

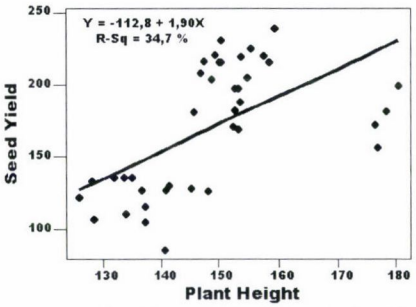



*Fig. 1.* Significant (**P<0.01) relationship between seed yield and plant height in synthetic genotypes and standard varieties (over mean of 3 years)

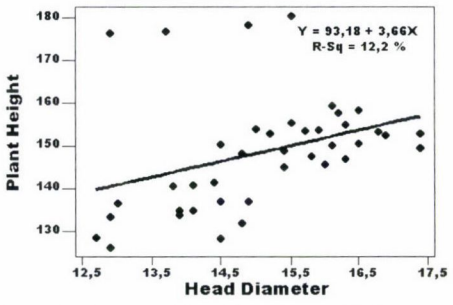



*Fig. 2.* Significant (**P<0.01) relationship between seed yield and head diameter in synthetic genotypes and standard varieties (over mean of 3 years)

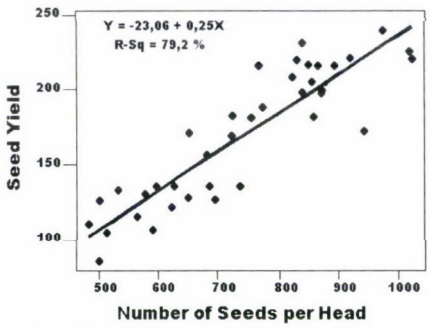



*Fig. 3.* Significant (**P<0.01) relationship between seed yield and number of seeds per head in synthetic genotypes and standard varieties (over mean of 3 years)

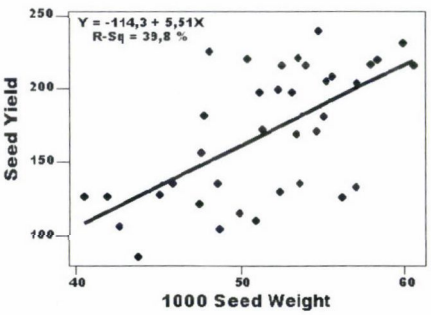



*Fig. 4.* Significant (**P<0.01) relationship between seed yield and 1000-seed weight in synthetic genotypes and standard varieties (over mean of 3 years)

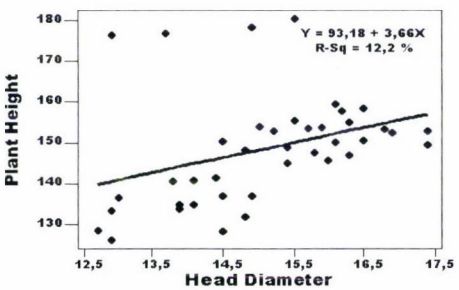



*Fig. 5.* Significant (*P<0.05) relationship between plant height and head diameter in synthetic genotypes and standard varieties (over mean of 3 years)

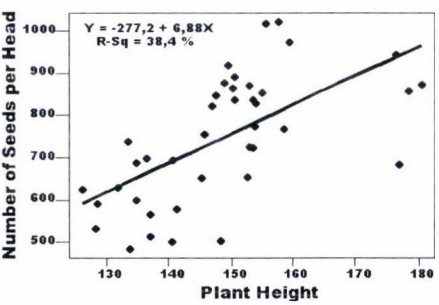



*Fig. 6.* Significant (**P<0.01) relationship between number of seeds per head and plant height in synthetic genotypes and standard varieties (over mean of 3 years)

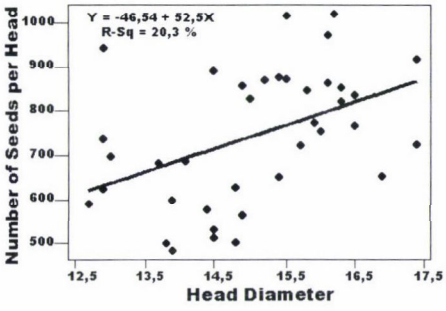



*Fig. 7.* Significant (**P<0.01) relationship between number of seeds per head and head diameter in synthetic genotypes and standard varieties (over mean of 3 years)

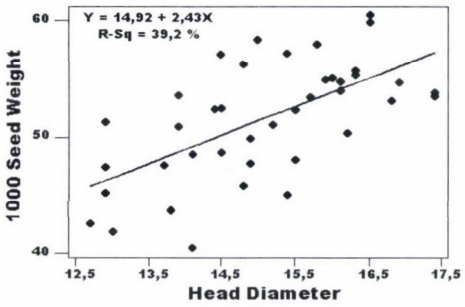



*Fig. 8.* Significant (**P<0.01) relationship between 1000-seed weight and head diameter in synthetic genotypes and standard varieties (over mean of 3 years)

In the study, seed yield was taken as a dependent variable and plant height, head diameter, number of seeds per head and 1000-seed weight as independent variables. All the data were subjected to path analysis to determine the direct and indirect effects of the independent variables on the seed yield (Dewey and Lu, 1959). The results of path coefficient analysis (Table 2) revealed that the number of seeds per head exerted the greatest direct positive effect on the seed yield, followed by 1000-seed weight. These direct effects were seen to be stable over the years. Plant height exhibited a negative direct effect, while head diameter had a low positive direct effect on seed yield. The direct effect made up 80.8% of the positive significant correlation (r=+0.890) between the number of seeds per head and the seed yield. However, the number of seeds per head had a positive indirect effect through the 1000-seed weight (10.1%) and head diameter (8.5%) on seed yield. It was also reported in earlier studies that the number of seeds per head exerted the greatest direct positive effect on the seed yield (Rana et al., 1991; Alvarez et al., 1992; Punia and Gill, 1994). On the other hand, Patil et al. (1996) reported that the number of seeds per head also had the greatest indirect effect through other characters on the seed yield.

The positive and significant association between seed yield and 1000-seed weight (r=+0.631) was 50.6% a direct effect, but 1000-seed weight also had a positive indirect effect of 32.4% on seed yield through the number of seeds per head and 16.7% through the head diameter. In close agreement with these findings, Niranjana and Shambulingappa (1989), Patil et al. (1996) and Lal et al. (1997) reported that 1000-seed weight directly affected the seed yield.

Averaged over three years, the direct effect of head diameter on seed yield was positive but weak (r=+0.1689), and made up only 24.0% of the total effect, while 46.9% of the positive significant correlation (r=+0.696) between head diameter and seed yield could be attributed to the indirect effect (r=+0.3306) of head diameter through the number of seeds per head. Head diameter also had a positive indirect effect (+0.2015) on seed yield through the 1000-seed weight.

Singh and Labana (1990), Hussein et al. (1995) and Doddamani et al. (1997) reported that head diameter had the greatest direct effect on seed yield.

The direct effect of plant height on the seed yield was negative and weak (r=–0.0075). However, 74.6% of the positive significant correlation (r=+0.589) between plant height and seed yield was the result of an indirect effect (r=+0.4507) through the number of seeds per head.

*Table 2*
Path coefficients – Direct effects (in diagonal line) and indirect effects

| Character | Plant height | | Head diameter | | No. of seeds/head | | 1000-seed weight | |
|---|---|---|---|---|---|---|---|---|
| | P | % | P | % | P | % | P | % |
| Plant height | 0.1814 | 49.9 | 0.0058 | 1.6 | 0.1716 | 47.3 | 0.0044 | 1.2 |
| | 0.0409 | 7.9 | 0.0044 | 0.8 | 0.2588 | 49.6 | 0.2176 | 41.7 |
| | –0.0251 | 4.8 | –0.0070 | 1.3 | 0.4225 | 81.0 | 0.0671 | 12.9 |
| | –0.0075 | 1.3 | 0.0607 | 10.0 | 0.4507 | 74.6 | 0.0853 | 14.1 |
| Head diameter | 0.0071 | 1.5 | 0.1490 | 31.2 | 0.0197 | 4.1 | 0.3018 | 63.2 |
| | 0.0062 | 0.9 | 0.0292 | 4.3 | 0.5137 | 75.2 | 0.1339 | 19.6 |
| | –0.0106 | 4.5 | –0.0167 | 7.1 | 0.1690 | 71.4 | 0.0403 | 17.0 |
| | –0.0027 | 0.4 | 0.1689 | 24.0 | 0.3306 | 47.0 | 0.2015 | 28.6 |
| No. of seeds/head | 0.0417 | 4.5 | 0.0039 | 0.4 | 0.7466 | 80.5 | –0.1350 | 14.6 |
| | 0.0135 | 1.5 | 0.0192 | 2.0 | 0.7818 | 83.4 | 0.1232 | 13.1 |
| | –0.0149 | 1.8 | –0.0040 | 0.5 | 0.7125 | 85.7 | 0.1003 | 12.0 |
| | –0.0047 | 0.5 | 0.0768 | 8.6 | 0.7269 | 80.8 | 0.0910 | 10.1 |
| 1000-seed weight | 0.0014 | 0.2 | 0.0799 | 9.7 | –0.1790 | 21.7 | 0.5634 | 68.4 |
| | 0.0027 | 4.1 | 0.0118 | 1.8 | 0.2921 | 44.2 | 0.3297 | 49.9 |
| | –0.0049 | 0.9 | 0.0020 | 0.3 | 0.2077 | 37.2 | 0.3438 | 61.6 |
| | –0.0020 | 0.3 | 0.1059 | 16.7 | 0.2057 | 32.4 | 0.3215 | 50.6 |

P = Path coefficient values in 1995, 1996, 1997 and mean of 3 years, downward, respectively;
% = Percentage of direct effects

## Conclusions

In the present study, correlation and path analysis were applied to seed yield, plant height, head diameter, number of seeds per head and 1000-seed weight in a population consisting of Syn 1s, Syn 0s and two commercial cultivars of sunflower. Simple correlation coefficients between seed yield and four traits were found to be positive and highly significant. In sunflower, seed yield per unit area depends on the plant number (head number) per unit area and the seed weight per plant (head). Highly positive correlations were thus expected between the seed yield and both the number of seeds per head and the 1000-seed weight. In the present material, plant height and head diameter also gave high correlations with seed yield.

Path analysis revealed that the direct effect of number of seeds per head on seed yield was positive and was the highest in the study. This character was followed by 1000-seed weight and head diameter. Plant height did not affect seed yield directly, but its indirect effect through the number of seeds per head was unexpectedly found to be highly significant.

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# EFFECT OF RESIDUE RECYCLING ON A CROPPING SYSTEM BASED ON FODDER SORGHUM

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A farming system experiment was conducted under dryland conditions in the western zone of Tamil Nadu with cropping, agroforestry, pigeons, goats, buffaloes and farm pond as the enterprise combination from July 2000 to March 2002. The cropping system of sorghum (fodder) + cowpea (fodder) followed by chickpea + coriander was supplied with either composted goat manure or composted buffalo manure at 100 or 75% of the production levels in order to investigate the effect of the source and amount of manures from the livestock enterprises linked in the system on cropping. The application of composted buffalo manure at 100% production level resulted in higher yield attributes, yield and returns in the second year, though the application of the recommended dose of fertilizers gave higher values for the same parameters in the first year, due to the buildup of nutrients over time. Higher yields of chickpea and coriander also indicated the favourable residual effect of the organic manures. This treatment ranked best with the highest benefit to cost ratio among the treatments imposed.

**Key words**: integrated farming system, enterprise combination, recycling, manure

## Introduction

Dryland agriculture in India is always a challenge since crop production depends on monsoon rains. Much of the land under agriculture is held by small or marginal farmers who are resource-poor, and cultivation is done under conditions of low or no fertilizer application. Conventional dry farming is risky, with a number of biophysical and socioeconomic constraints limiting the average productivity of dryland crops. This has served as the driving force for the development of farming systems research. An enterprise combination of crops and animals on the farm would offset the risk associated with crop production alone. Though the types of interaction are varied, their implications are especially significant to the community of small or marginal farmers

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(Dibissia and Peters, 1999; Devendra, 2000). However, it is essential to integrate suitable enterprises at the optimum level so as to capture greater benefits. Studies on integrated farming systems for different agroclimatic regions and on farm situations have been carried out by a number of research workers in India (Pandey, 1988; Salam et al., 1991; Saran et al., 1993; Devasenapathy et al., 1995; Senthilvel et al., 1998). The recycling of resources and residues from one enterprise to the other forms the basis of ecological stability in these systems. Still, there is insufficient literature on the effect of the enterprises integrated on cropping in particular. From a study on the integrated farming systems, this paper attempts to document the effect of the linked animal enterprises on a cropping system based on sorghum fodder, where the manure was recycled as the only source of nutrients.

## Materials and methods

The investigation was carried out as a farming system experiment from July 2000 to March 2002 in two one-year cycles at the dryland research farm of the Tamil Nadu Agricultural University, Coimbatore. The soil at the experimental site was vertisol. Rainfall during the cropping season was 290.4 and 372.1 cm in 2000–01 and 2001–02, respectively. The enterprise combination included cropping on 0.80 ha, agroforestry on 0.10 ha, pigeons (10 pairs) on 0.01 ha, goats (5:1 female : male, Tellicherry breed) and/or buffaloes (2 milking and 1 calf, local breed) on 0.05 ha and farm pond on 0.04 ha, so as to collectively represent a 1 ha farm area. The manure produced from the goat and buffalo units was recycled to cropping after composting at 100% and 75% of the production level.

The fodder-based cropping system of sorghum (fodder) + cowpea (fodder)/chickpea + coriander was cultivated during the 2000–01 and 2001–02 season in a randomised block design with 4 replications. The treatments included: no manure ($S_0$), recommended dose of NPK through fertilizers ($S_1$), composted buffalo manure at 100% of the production level ($S_2$), composted buffalo manure at 75% of the production level ($S_3$), composted goat manure at 100% of the production level ($S_4$) and composted goat manure at 75% of the production level ($S_5$). The levels of manure added and the nutrient content are presented in Table 1. The effect of the source and amount of manure obtained from the linked livestock enterprises on cropping within a farming system was studied. The crop growth and yield attributes were recorded along with the yield and statistically analysed. The economic efficiency was also calculated.

*Table 1*
Nutrient content and amount of organic manures used

| Particulars | Buffalo manure (Composted) | Goat manure (Composted) |
|---|---|---|
| Nutrient content | | |
| 1. N (%) | 0.87 | 1.82 |
| 2. P (%) | 0.39 | 0.95 |
| 3. K (%) | 0.52 | 0.92 |
| 4. Cu (ppm) | 24 | 31 |
| 5. Mn (ppm) | 271 | 352 |
| 6. Fe (ppm) | 360 | 570 |
| 7. Zn (ppm) | 162 | 150 |
| Quantity added (kg $ha^{-1}$) | | |
| 100% of the production level | 7250 | 1200 |
| 75% of the production level | 5400 | 900 |

## Results and discussion

*Effect on yield attributes*

Crop yield components varied significantly in response to the source of nutrient and amount of manure added (Table 2).

Sorghum (fodder): The single plant weight that determines the yield of fodder sorghum varied significantly between the treatments imposed. The highest weight was obtained with the application of the recommended dose of NPK fertilizers, followed by the application of composted buffalo manure at 100% of the production level in the year 2000–01. This could be attributed to the immediate availability of the nutrients from inorganic fertilizer. However, in 2001–02, these two treatments gave comparable plant weight. The lowest single plant weight in the first year was observed with the application of composted goat manure at 75% of the the production level, which was comparable with no fertilizer. However, during the second year, the application of composted goat manure at the 75% level was significantly superior to no manure. The single plant weight recorded with the application of composted buffalo manure at 75% production level was significantly inferior to its application at the 100% level in both years, due to the reduced quantity of nutrients available at the lower application rates. Toor et al. (2001) reported significantly higher available N with increasing rates of manure application.

Cowpea (fodder): The application of composted buffalo manure at the 100% level favourably influenced the single plant weight, giving weights comparable with the recommended NPK fertilizers. Cowpea, being a legume crop, was able to fix atmospheric N to supplement its needs in addition to the nutrients available from composted buffalo manure. However, imposing a 25% reduction in the composted buffalo manure application reduced the single plant weight. Higher plant weight was recorded with composted goat manure at 100% the production level and was comparable with composted goat manure at the 75% level. Though the nutrient content per kg of composted goat manure was higher compared to composted buffalo manure, the lower quantities of manure generated from the goat unit in the system resulted in fewer nutrients for the crops, resulting in lower growth and yield attributes. The lower level of plant weight was recorded with no manure. This character expressed a similar trend during the second year of study.

Chickpea: The number of pods per plant and test weight were favourably influenced by the application of organic manures to the preceding crop of fodder sorghum + fodder cowpea. Higher values of the parameters were recorded with composted buffalo manure at 100% of the the production level, followed by comparable results for the application of the recommended NPK fertilizers and composted buffalo manure at the 75% level. The variations in the level of composted goat manure applied did not influence the number of pods per plant or test weight, but resulted in higher test weight than no manure.

*Table 2*
Yield attributes of crops in the sorghum (fodder) + cowpea (fodder)/chickpea + coriander system

| Treatments* | Sorghum (fodder) | | Cowpea (fodder) | | Chickpea | | | |
|---|---|---|---|---|---|---|---|---|
| | Single plant weight (g) | | Single plant weight (g) | | No. of pods plant$^{-1}$ | | Test weight (g) | |
| | 2000–01 | 2001–02 | 2000–01 | 2001–02 | 2000–01 | 2001–02 | 2000–01 | 2001–02 |
| $S_0$ | 109.1 | 123.8 | 57.0 | 77.3 | 5.65 | 4.86 | 29.12 | 29.05 |
| $S_1$ | 144.4 | 182.3 | 68.3 | 94.2 | 9.41 | 7.93 | 32.34 | 31.47 |
| $S_2$ | 133.7 | 180.5 | 66.3 | 94.7 | 10.03 | 8.54 | 32.63 | 31.73 |
| $S_3$ | 129.2 | 164.2 | 63.5 | 86.3 | 9.29 | 8.41 | 32.19 | 31.55 |
| $S_4$ | 118.0 | 145.4 | 60.4 | 82.5 | 8.80 | 7.64 | 30.41 | 30.28 |
| $S_5$ | 111.8 | 132.9 | 59.8 | 81.1 | 8.78 | 7.57 | 29.97 | 29.70 |
| SEd | 1.87 | 2.09 | 1.07 | 1.37 | 0.20 | 0.19 | 0.27 | 0.26 |
| CD (P=0.05) | 4.18 | 4.66 | 2.38 | 3.06 | 0.45 | 0.43 | 0.61 | 0.59 |

* For treatments see Materials and methods

*Yield*

The yield of the crops in the cropping system is indicated in Table 3.

Sorghum (fodder): Each treatment studied was significantly different from the other in the first year of study, with the application of the recommended NPK fertilizers producing the highest green fodder yield. Though this trend continued in the second year, composted buffalo manure at the 100% level gave a comparable green fodder yield as a result of the organic and nutrient buildup of the soil, leading to better soil health. Although the slow mineralization of organic manures resulted in low initial nutrient status in the organic manure treatments, there was a buildup of nutrients over time.

Cowpea (fodder): Organic manure application exerted a favourable influence on the green fodder yield of cowpea. The green fodder yield recorded with the application of the recommended level of NPK fertilizers was comparable with that obtained with the application of composted buffalo manure at the 100% level. The application of composted goat manure at the 75% level produced the lowest green fodder yield among the treatments receiving organic manure, but was higher than no manure and comparable to composted goat manure at the 100% level. A similar trend was observed in the second year. Though the nutrient content was higher in goat manure, the lower amounts added resulted in lower yields.

Chickpea: The organic manures applied to fodder sorghum + fodder cowpea influenced the seed yield of succeeding chickpea in both years. The application of composted buffalo manure at 100% of the production level gave the highest seed yield in both years. However, in the second year of study, composted buffalo manure at the 75% level also resulted in a comparable seed yield of chickpea. The slow, phased release of organic and mineral N nutrients from the organic manures left in the soil and the N-fixing capacity of chickpea might have contributed to this increase. Similar observations were made by Powlson et al. (1992) and Addiscott and Dexter (1994). The seed yield recorded

with the application of composted goat manure at the 100% level was comparable to that of the 75% level, but was higher than no manure application. The effect of composted goat manure application followed a similar trend in both the years.

Coriander: Organic manures were found to have a favourable residual effect on the succeeding coriander crop grown with chickpea in this experiment. The highest green mass and seed yield of coriander were observed with composted buffalo manure at 100% of the production level in both years of the study. Comparable green mass was recorded with composted buffalo manure at the 75% level and the recommended NPK fertilizers. Composted goat manure application resulted in lower coriander seed yield and green mass. However, it was higher than that recorded with no manure application. As stated earlier, the lower quantity of manure obtained from the goat enterprise meant a poorer supply of nutrients to the crop. This resulted in lower yield after the application of composted goat manure at either level.

Sorghum fodder-equivalent yield: The total yield of the intercropping system, expressed as sorghum fodder-equivalent yield (Table 3), was significantly higher (119.12 and 110.51 t/ha) with the application of composted buffalo manure at 100% of the production level in both years of the study, being 2.9 t/ha higher in the first year and 5.3 t/ha in the second year compared to the second best yield recorded. The total yield obtained with composted buffalo manure at the 75% level and the application of the recommended dose of NPK fertilizers were on par in the second year though they varied significantly in the first year. No manure application led to the lowest sorghum fodder-equivalent yield. The higher yield obtained for the crops in the system with the application of composted buffalo manure at the 100% level was reflected as a higher sorghum fodder-equivalent yield.

*Table 3*

Yield of crops (kg/ha) and sorghum fodder-equivalent yield (Equiv.; t/ha) in the sorghum (fodder) + cowpea (fodder)/chickpea + coriander system

| Treatments* | Sorghum | | Cowpea | | Chickpea | | Coriander | | | | Equiv. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Green fodder | | Green fodder | | Seed | | Green mass | | Seed | | | |
| | A | B | A | B | A | B | A | B | A | B | A | B |
| $S_0$ | 16247 | 18307 | 4229 | 5714 | 539 | 435 | 341 | 234 | 81 | 56 | 71.09 | 64.21 |
| $S_1$ | 21463 | 27035 | 5081 | 6985 | 993 | 811 | 522 | 393 | 150 | 98 | 116.19 | 105.25 |
| $S_2$ | 19877 | 26786 | 4912 | 7023 | 1043 | 858 | 537 | 426 | 163 | 115 | 119.12 | 110.51 |
| $S_3$ | 18516 | 24453 | 4736 | 6427 | 981 | 841 | 514 | 402 | 152 | 101 | 112.07 | 104.10 |
| $S_4$ | 17515 | 21608 | 4546 | 6123 | 920 | 774 | 483 | 355 | 137 | 89 | 104.78 | 94.46 |
| $S_5$ | 16666 | 19355 | 4445 | 6013 | 898 | 757 | 462 | 313 | 130 | 84 | 101.23 | 89.58 |
| SEd | 170 | 235 | 77 | 84 | 11 | 13 | 6 | 8 | 4 | 4 | 0.72 | 0.69 |
| $CD_{P=0.05}$ | 379 | 521 | 172 | 187 | 25 | 29 | 12 | 19 | 9 | 9 | 1.51 | 1.44 |

* For treatments see Materials and methods; A. 2000–01; B: 2001–02

*Economic analysis*

The highest net returns and benefit to cost (B:C) ratio of 2.26 was observed with the application of composted buffalo manure at 100% of the production level, when the data of both years were pooled (Table 4). This was mainly because of the better growth and yield parameters and the consequent high yield recorded in the crops due to the application of composted buffalo manure. The recycling of organic manures produced on the farm itself reduced the cost of cultivation, as there was no investment in the purchase of fertilizers. The application of composted goat manure at the 100% level also resulted in a higher B:C ratio on account of the lower cost of cultivation and better yields. However, the superiority of the application of composted buffalo manure at the 100% level to the sorghum (fodder) + cowpea (fodder)/chickpea + coriander system was brought out clearly by this experiment.

Thus, the results of the two-year study suggest that the application of composted buffalo manure at 100% of the production level, when 2 buffaloes and 1 calf are integrated in the system, produces higher yield and economic benefits in a fodder sorghum-based cropping system. The recycling of manure from the livestock enterprises integrated with cropping in the farming system approach under dryland conditions adds valuable plant nutrients for plant growth and sustains the fertility of the soil base, besides increasing productivity.

*Table 4*
Economic analysis of sorghum (fodder) + cowpea (fodder) – chickpea + coriander system

| Treatments* | Cost of cultivation** | Gross returns** | Net returns** | B:C ratio |
|---|---|---|---|---|
| $S_0$ | 14535 (227) | 23263 (364) | 8728 (137) | 1.60 |
| $S_1$ | 17577 (275) | 38132 (597) | 20555 (322) | 2.17 |
| $S_2$ | 17545 (274) | 39550 (619) | 22005 (344) | 2.26 |
| $S_3$ | 16905 (264) | 37240 (583) | 20335 (318) | 2.21 |
| $S_4$ | 15455 (241) | 34325 (537) | 18870 (295) | 2.22 |
| $S_5$ | 15355 (240) | 32883 (514) | 17528 (274) | 2.15 |

* For treatments see Materials and methods; ** in Rupees $ha^{-1}$, approx. € equivalent in brackets

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# EFFECTS OF POST-EMERGENT HERBICIDES ON *Trichoderma harzianum,* A POTENTIAL BIOCONTROL AGENT AGAINST *Sclerotinia sclerotiorum* IN SOYBEAN CROPPING

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*Trichoderma harzianum* is a potential biocontrol agent against *Sclerotinia sclerotiorum* in soybean. Information is needed on the compatibility of this biocontrol agent and the post-emergent herbicides used in soybean cropping.

Haloxyfop R Methyl (EC 10.4%), Glyphosate (SL 48%), Imazamox (WG 70%) and Imazethapyr (SL 10%) were evaluated for their effects on the mycelial growth of *T. harzianum* on *in vitro* agar plates. Glyphosate (2000 ppm), Imazethapyr (500 and 250 ppm) and Haloxyfop R Methyl (1000, 500 and 100 ppm) reduced the mycelial growth of *T. harzianum.* Imazamox had no effect at any concentration.

Subsequently, all the herbicides were assessed for their effect on soil populations of *T. harzianum.* Greenhouse assays conducted with non-sterile soil inoculated with *T. harzianum* and a specific herbicide were sampled before pesticide application and after 30 days. The number of colony forming units per gram of soil (c.f.u./g of soil) was evaluated with a soil dilution technique using *Trichoderma* selective medium (TSM). No detrimental effect was revealed.

**Key words:** biocontrol agent, population density, *Trichoderma harzianum*, soybean stem decay, post-emergent herbicides

## Introduction

Stem decay caused by *Sclerotinia sclerotiorum* is one of the main diseases affecting soybean in Argentina (Distéfano de Vallone and Giorda, 1997; Ploper, 1999). The primary source of inoculum is the sclerotium that survives in the soil. The destruction or inactivation of sclerotia can reduce primary inoculum and might provide a way of controlling this disease (del Río et al., 2002; Budge and Whipps, 2001).

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Many researchers have obtained effective biological control of Sclerotinia diseases using species of *Trichoderma* as mycoparasites of the sclerotia of *S. sclerotiorum* (Knudsen et al., 1991; Homechin, 1983; Mitidieri and Scandiani, 1989; Illipronti and Machado, 1993; Inbar et al., 1996; Mónaco et al., 1998; Bae et al., 2001; Mc Lean et al., 2001; Elad et al., 2002).

The potential utility of *Trichoderma* as a biocontrol agent depends largely on its compatibility with agricultural chemicals or other control practices (Papavizas, 1985; Pereira et al., 1996; Nath et al., 2004; Chakraborty et al., 2003). Herbicides are reported to affect the growth and sporulation of *Trichoderma* spp. in *in vitro* tests (Dluzniewska, 2003; Macek and Lesnik, 1994; Jayaraj and Radhakrishnan, 2000a; Sesan, 2002; Desai and Srikant, 2004). A variable effect of Glyphosate on soil microflora has been shown in several reports, including reduced bacterial, actinomycete and fungal populations in forest soils (Gorlach-Lira et al., 1997), increased populations of soil actinomycetes and fungi (Araújo et al., 2003), increased soil biomass (Haney et al., 2002) or no long-term change in microbial populations (Busse et al., 2001). Information about the effects of herbicides on the survival of *Trichoderma* spp. in soil was given by Rao and Divakar (2002), who reported some inhibitory effects with Butachlor and 2,4 D on *T. viride*, and by Jayaraj and Radhakrishnan (2000b) with Pendimethalin on *T. harzianum.*

Previous research by Mónaco et al. (1998) was initiated with *T. harzianum* (strain ThS12) because of its ability to parasitise the sclerotia of *S. sclerotiorum*. If this strain is to be used for the biological control of soybean stem decay, information on the compatibility of the biological control agent and the agricultural chemicals used in soybean cropping is needed.

The objective of this research was to evaluate the effect of the post-emergent herbicides used in protected soybean crops in Argentina on the mycelium growth on *in vitro* agar plates and on soil populations of *T. harzianum* (strain ThS12).

## Materials and methods

*Isolates and cultures*

*Trichoderma harzianum* (strain ThS12), used in this study, has been identified as a promising biocontrol agent of *Sclerotinia sclerotiorum* due to its ability to parasitise sclerotia (Mónaco et al., 1998). Isolates were grown on 2% potato dextrose agar (PDA) and stored at 5°C.

*Herbicides*

The post-emergent herbicides used are listed in Table 1 (Anonymous, 2001; Tomlin, 1994).

T. harzianum *mycelial growth on PDA*

Various formulations of the herbicides were analysed on PDA to investigate their effect on the mycelial growth of *T. harzianum.*

*Table 1*
Names and application conditions of the post-emergent herbicides used

| Common name | Chemical name | Rate of application | Type of formulation and concentration (%) |
|---|---|---|---|
| Haloxyfop R Methyl | -2-(4-((3- chloro -5(trifluoromethyl)-2-pyridinyl)oxy) phenoxy) propanoic acid methyl) | 1.4 l/ha | EC 10.4 |
| Glyphosate | N-(phosphonomethyl) glycine | 6 l/ha | SL 48 |
| Imazamox | 2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-5-methoxymethylnicotinic acid | 70 g/ha | WG 70 |
| Imazethapyr | 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-5-ethyl-3-pyridinecarboxylic acid | 0.8 l/ha | SL 10 |

Doses of herbicides were added to 9 ml of molten PDA to give final concentrations of 2000, 1000, 100 and 10 ppm of Glyphosate, 1000, 500, 100 and 10 ppm of Haloxyfop R Methyl, 500, 250, 100 and 10 ppm of Imazethapyr and 50, 25, 10 and 5 ppm of Imazamox. The highest concentration of each herbicide corresponded to a tenth part of the field rate recommended for Argentina. For the controls, 1 ml of distilled water was added to 9 ml of molten PDA.

The molten PDA was poured into 9-cm diameter Petri dishes and allowed to cool. Cores of mycelia (6 mm diameter) from the leading edge of *T. harzianum* cultures on PDA were placed in the centre of the Petri dishes containing the herbicide treatments and controls.

The plates were incubated at 25°C in the dark. After five days of incubation, the diameter of the fungus colonies was measured. Each plate represented one experimental unit. Each treatment consisted of four replications and the experiment was conducted twice.

T. harzianum *soil population studies*

Inoculum was prepared by growing the fungus on a substrate containing bran wheat, sand and distilled water (2:1:2 w/w/v). The substrate was autoclaved for three consecutive days at 120°C for 20 minutes. Incubation was carried out in an incubator at 25°C in darkness for 20 days.

Silt loam soil was used (clay 21.2%, lime 56%, sand 22.8%; pH: 6.4; C: 1.95%; OM %: 3.35, moisture %: 24.70%). The pots were filled with 1 kg dry soil and amended with wheat bran colonised by *T. harzianum*. The culture medium was crumbled in the soil until a homogeneous mixture was obtained.

Herbicides were applied as a suspension in water, adjusted to the recommended rate of application to the soil surface.

For each treatment the fungus and a selected herbicide were incorporated into the soil in each pot. The controls received only the fungus.

There were five treatments: 1) *T. harzianum* with Haloxifop R Methyl, 2) *T. harzianum* with Glyphosate, 3) *T. harzianum* with Imazethapyr, 4) *T. harzianum* with Imazamox and 5) control (*T. harzianum* alone). Each pot represented one experimental unit. Each treatment consisted of four replications and the experiment was conducted twice.

The pots were kept in the greenhouse at a minimum temperature of 10°C and a maximum average temperature of 28°C for 30 days. Soil moisture was adjusted to 80% of field capacity by watering the pots periodically.

The treatments were arranged in a completely randomised design.

Soil samples (50 g) were collected from each pot before pesticide application and after 30 days.

The samples were dried at room temperature and then stored at 5°C for estimating the population density of the fungus.

The population density of the fungus was determined with a soil dilution technique using *Trichoderma* selective medium (TSM) (Elad et al., 1981). Six plates for each sample were incubated at 25 ± 2°C for a week in darkness. The number of colony forming units per gram of soil (c.f.u./g) was determined.

*Data analysis*

The data from all the experiments were subjected to analysis of variance and the means were compared using Tukey's test at 95% probability.

The number of c.f.u./g of soil of *T. harzianum* from each pot at 30 days was transformed to a percentage of the c.f.u./g of the initial soil (before application of herbicides), taken as 100%.

The percentage values were transformed following the formula:

$$Z = \text{arcsine (percentage)}\ ½$$

Since the results of the two experiments under similar conditions were similar, data from only one experiment are presented.

## Results and discussion

### T. harzianum *mycelial growth on PDA*

The results of investigations into the effect of selected herbicides on *T. harzianum* fungus growth are given in Table 2.

The experiments showed that the effect of the herbicides on the mycelial growth of *T. harzianum* depended on the herbicide and its concentration. Glyphosate (2000 ppm), Imazethapyr (500 and 250 ppm) and Haloxyfop R Methyl (1000, 500 and 100 ppm) reduced the mycelial growth of *T. harzianum.* Imazamox had no effect at any concentration. Furthermore, *T. harzianum* did not sporulate on medium containing 2000 ppm of Glyphosate.

Similar results showing the unfavourable effect of herbicides on the mycelial growth of fungi of the genus *Trichoderma* were obtained by Dluzniewska (2003), who found that the herbicides Afalon 450 SC (45% linuron) and Racer 250 WP (25% fluorochloride) inhibited the growth of *T. pseudokoningii, T. viride* and *T. harzianum* by 67% and 89%, respectively, in *in vitro* tests.

Macek and Lesnik (1994) reported that the herbicides Triasulfuron (Logran), Triasulfuron + Fluoroglycofen (Statis) and Primisulfuron (Tell) had various effects on *T. longibrachiatum* in potato dextrose agar tests. All the herbicides inhibited the mycelial growth of *T. longibrachiatum* at field concentrations, but Tell and Statis stimulated growth at medium and low concentrations. Desai and Srikant (2004) found that the herbicide Glyphosate had a very inhibitory effect on *T. harzianum* development.

*Table 2*
Influence of herbicides on the mycelium growth of *T. harzianum* (in cm, 5th day)

| | Herbicide | Concentration | | | | |
|---|---|---|---|---|---|---|
| | | C1 | C2 | C3 | C4 | Control |
| Growth (cm) | Glyphosate | 2000 ppm | 1000 ppm | 500 ppm | 100 ppm | 0 ppm |
| | | 4.2 a | 9.0 b | 9.0 b | 9.0 b | 9.0 b |
| | Haloxyfop R Methyl | 1000 ppm | 500 ppm | 100 ppm | 10 ppm | 0 ppm |
| | | 5.0 a | 5.1 a | 6.0 b | 9.0 c | 9.0 c |
| | Imazethapyr | 500 ppm | 250 ppm | 100 ppm | 10 ppm | 0 ppm |
| | | 6.0 a | 6.9 a | 9.0 b | 9.0 b | 9.0 b |
| | Imazamox | 50 ppm | 25 ppm | 10 ppm | 5 ppm | 0 ppm |
| | | 9.0 a | 9.0 a | 9.0 a | 9.0 a | 9.0 a |

Values are the means of four replicates; Means followed by the same letters are not significantly different at the P= 0.05 level

The sporulating and cellulase-producing ability of *T. harzianum* was significantly reduced in *in vitro* tests by the herbicides Alachlor, Butachlor, 2,4-D, Oxyfluorfen and Pendimethalin, especially at higher concentrations (Jayaraj and Radhakrishnan, 2000a).

T. harzianum *population studies*

No significant reduction in the *T. harzianum* population was observed in soil treated with post–emergent herbicides compared with the untreated control (Fig. 1).

Among the herbicides tested, Glyphosate is one of the most widely used because of its effectiveness against many weeds. A high percentage (95–98%) of the soybean acreage in Argentina is planted with transgenic glyphosate-tolerant soybean cultivars (Anonymous, 2002). Farmers adopted this new technology due to the efficacy of Glyphosate against a wide range of weed species. Variable effects of Glyphosate on soil microflora have been shown by Gorlach-Lira et al. (1997), Araújo et al. (2003), Haney et al. (2002) and Busse et al. (2001). The present results are in line with those reported by Busse et al. (2001), who found that Glyphosate was toxic to bacteria and fungi when grown in soil-free media, but this toxicity was not expressed when Glyphosate was added directly to soil. These authors suggest that artificial medium assays are of limited relevance in predicting Glyphosate toxicity to soil organisms and that field rate applications of Glyphosate should have little or no effect on soil microbial communities. The differences between artificial media and field soil are believed to be due to the polar nature of the Glyphosate molecule and its adsorption to soil particles.

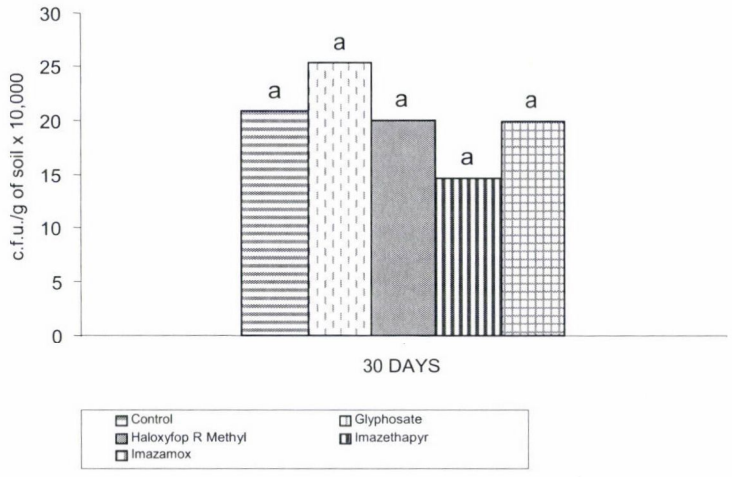



*Fig. 1. Trichoderma harzianum* population in soil in the presence of herbicides

Imazethapyr and Haloxyfop R Methyl behaved similarly to Glyphosate and are also weakly to moderately adsorbed on the soil (Ahrens, 1994). These results are also in agreement with Mc Lean et al. (2001), who stated that it is unlikely that the level of direct contact observed between fungus and pesticide in the *in vitro* assay would occur in a field environment, given the strong buffering capacity of the soil.

*In vitro* agar plate experiments are useful to determine the potential tolerance of fungi to pesticides, but assessing the compatibility between a biocontrol agent and pesticides in natural environments would provide a more reliable measure of compatibility.

The development of Integrated Pest Management requires knowledge of the impact of a selected pesticide not only on its intended target but also on other species, including beneficial microorganisms. *T. harzianum* (THS12) has antagonistic properties against *S. sclerotiorum,* as has been demonstrated in previous research (Mónaco et al., 1998). The results of these investigations show that this strain can survive in soil treated with Glyphosate and other selected post-emergent herbicides currently used to protect soybean crops in Argentina.

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# MORPHOLOGICAL ATTRIBUTES OF ORIENTAL SPRUCE [*Picea orientalis* (L.) LINK.] SEEDLINGS GROWN IN PEAT-BASED MEDIA AMENDED WITH NATURAL ZEOLITE

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This study was designed to investigate the influence of growth media consisting of different components on the morphological attributes of oriental spruce seedlings. Eighteen different combinations of Barma peat (BP), tea residue compost (CTR), fine pumice (FP), coarse pumice (CP), perlite (P) and zeolite (Z) were prepared as growth media.

The growth medium components did not significantly affect the shoot height (SH), root collar diameter (RCD), shoot:root ratio or dry root percentage (DRP) of 2-year-old seedlings. However, the root dry weight (RDW) and shoot dry weight (SDW) showed significant differences between the different growth media. The maximum SDW (3.244 g) was determined for a mixture of BP (0.5) + CTR (0.2) + CP (0.2) + Z (0.1), while a mixture of BP (0.6) + P (0.2) + Z (0.2) resulted in minimum SDW (1.593 g). In addition, the maximum RDW (1.824 g) was determined for the BP (0.5) + CTR (0.2) + CP (0.2) + Z (0.1) medium, while the BP (0.6) + CP (0.2) + Z (0.2) medium resulted in the lowest RDW (1.013 g). The addition of zeolite to the growth media increased the SDW and RDW of oriental spruce seedlings, so natural zeolite could be used as a substrate to replace pumice and perlite in nurseries in Turkey. Since Turkey has 45.8 billion tonnes of zeolite, its use in nurseries could reduce the costs significantly.

**Key words:** growth media, *Picea orientalis*, seedling attributes, zeolite

## Introduction

Planting nursery stock is both reliable and economical, and the potting medium used for growing seedlings in containers is very important to increase the desired morphological attributes of the seedlings. Various materials and mixtures can be used for seed germination and the rooting of cuttings. Most growth media consist of two or more different organic and inorganic components selected to provide specific physical, chemical or biological properties.

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Peat has a long history of use in greenhouse production even though it is not a readily renewable resource. Low-quality degraded peat (≥ H4 on the Von Post scale), which has small fibres, holds larger amounts of water and less air as compared to less degraded peat (Allaire et al., 2005). Puustjärvi (1973) reported that the structure of peat may be too coarse or too fine. However, the most common problem with the structure is that it is too fine. In that case, the water space becomes too large and the air space too small. In addition, the stability of the physical properties of the substrates is of primary concern for container-grown plants, because changes in these properties may negatively affect plant growth (Allaire-Leung and Caron, 1999). Moreover, organic substrates such as peat with low homogeneity and a high decomposition ratio create pathological problems and cause toxicity (Koksaldi, 1999).

The development of alternative peat substrates is necessary for three reasons: the limited resources of peat; the increasing pressure to use waste originating from human or industrial activities, and the economic necessity to use locally produced waste products (Guérin et al., 2001). The quality and stability of substrates are related to physical attributes such as particle size and geometry, pore-size distribution and arrangement, which influence water and gas storage, and exchange properties (Allaire-Leung and Caron, 1999).

The fertilization techniques normally used in container nurseries are not efficient for the management of nutrients. Moreover, it is difficult to control the fertility potential of substrates due to moisture and nutrient variability inside the container and the uncertainty of the relationship between the chemical composition of the substrate and the nutrient status of the plants (Lemaire et al., 1995). The reasons behind these difficulties are: (1) seedlings are grown in such small containers that slight changes in the physical (porosity, etc.) and chemical (acidity, electrical conductivity, etc.) properties of the growth medium could easily influence their growth, and (2) the low or moderate biostability of organic substrates, which release available nutrients inconsistently and vary in their chemical properties, such as pH, electrical conductivity (EC) and cation exchange capacity (CEC) as a consequence of the decomposition of the organic matter (Lemaire, 1997). Consequently, peat substrates are routinely amended with various materials such as large-particle-size perlite, rockwool, expanding clay, sand, wood bark, compost, polystyrene and polyurethane to obtain air-filled porosity (Nkongolo and Caron, 1999).

Zeolites, a naturally occurring mineral group consisting of about 50 mineral types, have long been considered as a good growth medium substrate due to thier good physical and chemical characteristics (Markovich et al., 1995). They have a rigid three-dimensional crystal structure with voids and channels of molecular size and high CEC arising from the substitution of Al for Si in the silicon oxide tetrahedral units that constitute the mineral structure (Pickering et al., 2002; Ayan, 2001; 2002a). Zeolite has many good features that make it very attractive as a growth medium for nursery use compared to other growth

medium types such as perlite, pumice or river sand. These features include high ammonium absorption capacity, good water and nutrient retention, and the slow release of N and P into the soil, similar to slow-release fertilizers (Koksaldi, 1999).

There is little research on the effects of growth media on the seedling attributes of oriental spruce and no research on zeolite as an inorganic component in growth media for container tree nurseries in Turkey. Turkey has 45.8 billion t of zeolite potential, so the use of zeolite for growing seedlings in nurseries could be economical. Thus, the objectives of this research were to investigate the influence of different growing media and mixtures (with and without zeolite) on the seedling morphology of containerized oriental spruce [*Picea orientalis* (L.) Link].

## Materials and methods

The study was carried out in the Of Forest Nursery in Trabzon, Turkey (elevation of 5 m) with seedlings of oriental spruce, a native, paleoendemic species of the Eastern Black Sea Region in Turkey. Oriental spruce is naturally distributed on approximately 350,000 hectares in Turkey and used as a commercial forest tree. Approximately 93,000 ha of pure oriental spruce stands are maintained by artificial regeneration and 140,000 ha of mixed stands by planting (Genç, 1995).

Mixtures of peat (BP), tea residue compost (CTR), perlite (P), fine pumice (FP), coarse pumice (CP) and natural zeolite (Z) were used as growth media. Peat was taken from the Barma Plateau at an altitude of 1800 m in Çaykara, Trabzon. These growth medium types were chosen because they provide better aeration and water permeability in pots, and absorb nutrients. Eighteen different volume combinations (%) (7:3, 5:2:3, 6:2:2, 7:2:1, 5:2:2:1) of the six different potting media with or without Z were used as growth media (Table 1). BP, which had 22% air capacity, 60% water-holding capacity and 88% total porosity, was used as the main additive material in the pots. The electrical conductivity of this material was 0.93 mS/cm, and the pH was between 4.9 and 6.0. BP was mainly of the sphagnum type with a small amount of grass mixture. The salt and lime contents were close to zero and the CEC was between 49 and 76 meq/100 g.

Pumice originating from Nevsehir consisted of 60–75% $SiO_2$, 13–15% $Al_2O_2$, 1–3% $Fe_2O_3$, 1–2% CaO, 1–2% MgO and 7–8% $Na_2O$–$K_2O$. It also had very low amounts of $TiO_2$, $SO_3$ and Cl and its pH ranged from 7 to 7.5 with a very low salt content.

The chemical composition of natural Z, originating from Manisa-Gordes, was 71.29% $SiO_2$, 13.55% $Al_2O_3$, 1.15% $Fe_2O_3$, 3.50% $K_2O$, 5.90% $H_2O$, 1.96 % CaO, 0.70% MgO, 0.60% $Na_2O$, 0.02% Ti, 0.04% Ag and 30 ppm B.

CTR was analysed after composting for 10–12 months and was found to have a C/N ratio of 6.69–14.52, total P of 12,780–17,798 ppm, exchangeable P of 2148–5489 ppm, total nitrogen of 1.753–3.72%, total water capacity of 544.71–679.00% and organic matter of 42.807–43.785%. The quantity of exchangeable and total $Mn^{++}$, $Al^{+++}$ and $Fe^{++}$ microelements in the CTR was also determined (Altun, 1988)

*Seedling production*

Seeds from the Kapıkoy province of Macka, Trabzon were sown in Enso-Finland Model Type containers (32 × 45 × 10 cm) in March using a seed drill. Three seeds were sown in each pot. The seedlings were thinned after 2 months to leave one seedling per pot. Sufficient seedlings were obtained in all the media, and no differences were observed between the media. The containers were kept in a greenhouse for two months after sowing, and then transferred to a shaded area for approximately one year for acclimatization. The seedlings were then kept under outdoor conditions until the end of the second growing season.

*Table 1*
Volume combinations of growth media used in pots for each treatment

| Growth medium symbols | Growth medium component (%) | | | | | |
|---|---|---|---|---|---|---|
| | BP (1–3 mm) | CTR | P | FP (2–4 mm) | CP (4–8 mm) | Z (Dust-size) |
| BP + P (7:3) | 70 | | 30 | | | |
| BP + FP (7:3) | 70 | | | 30 | | |
| BP + CP (7:3) | 70 | | | | 30 | |
| BP + CTR + P (6:2:2) | 60 | 20 | 20 | | | |
| BP + CTR + FP (6:2:2) | 60 | 20 | | 20 | | |
| BP + CTR + CP (6:2:2) | 60 | 20 | | | 20 | |
| BP + CTR + P (5:2:3) | 50 | 20 | 30 | | | |
| BP + CTR + FP (5:2:3) | 50 | 20 | | 30 | | |
| BP + CTR + CP (5:2:3) | 50 | 20 | | | 30 | |
| BP + P + Z (7:2:1) | 70 | | 20 | | | 10 |
| BP + P + Z (6:2:2) | 60 | | 20 | | | 20 |
| BP + FP + Z (7:2:1) | 70 | | | 20 | | 10 |
| BP + FP + Z (6:2:2) | 60 | | | 20 | | 20 |
| BP + CP + Z (7:2:1) | 70 | | | | 20 | 10 |
| BP + CP + Z (6:2:2) | 60 | | | | 20 | 20 |
| BP + CTR + P + Z (5:2:2:1) | 50 | 20 | 20 | | | 10 |
| BP + CTR + FP + Z (5:2:2:1) | 50 | 20 | | 20 | | 10 |
| BP + CTR + CP + Z (5:2:2:1) | 50 | 20 | | | 20 | 10 |

*Fertilization*

Fertilizer was applied following the recommendations of Richard and McDonald (1979) for the pH, nitrate and EC of the growth media (Table 2).

*Physical, chemical and biostability characteristics of the growth media*

Before sowing and fertilization, the growing medium samples were analysed for physical and chemical properties such as bulk density, water-holding capacity, specific gravity, porosity and air capacity (Tables 3 and 4). Bulk density was determined on 1000 $cm^3$ samples with 80% moisture content. The samples were oven-dried at 105°C for 24 h and weighed. To determine the water-holding capacity (%) 500 $cm^3$ samples were taken and put under 1 $g/cm^3$ pressure before wetting them in a tray for one night, after which the samples were oven-dried at 105°C for 24 h and weighed. The specific gravity was determined according to the picnometer method, and porosity was calculated using the following formula: Porosity (%) = (Specific gravity – bulk density) × 100 / Specific gravity. The air content was estimated as follows: Air content (%) = porosity (%) – water-holding capacity (%).

The organic matter content was determined by wet digestion (modified Walkley-Black Procedure) (Kalra and Maynard, 1991). Soil pH was measured with a combination glass-electrode in $H_2O$ (soil-solution ratio, 1:2.5) and CEC by saturating soil samples with $NH_4$ by leaching buffered $NH_4OAc$ solution (Kalra and Maynard, 1991). Phosphorus was determined according to the Bray 1 (dilute acid-fluoride) procedure (Kalra and Maynard, 1991). Exchangeable cations ($Na^+$, $Ca^{++}$, $Mg^{++}$, $K^+$) and micronutrient cations (Fe, Mn, Cu, Zn) were extracted from the neutral ammonium acetate solution and measured by atomic absorption spectrophotometry according to Kacar (1996). Electrical conductivity and ignition loss were determined following the procedure described by Kalra and Maynard (1991). Total nitrogen (%) was determined using the Kjeldahl method (Kjeltec Auto1030) (Kalra and Maynard, 1991). Biological stability was calculated as C/N.

*Table 2*
Application of fertilizers

| Fertilizer | Chemical content | Application time |
|---|---|---|
| Superex-9 | N 19% + P 5% + K 20% + microelements | At the beginning of the vegetation season |
| Superex-5 | N 11% + P 4% + K 25% + microelements | In the middle of the vegetation season |
| Superex-7 | N 0% + P 16% + K 20% + microelements | Before the end of the vegetation season |

*Table 3*
Initial physical properties of the growth media

| Growth medium symbols | Water capacity (% volume) | Air capacity (% volume) | Porosity (% volume) | Volume weight ($g/cm^3$) | Ignition loss (%) | Specific gravity ($g/cm^3$) | Organic matter (%) |
|---|---|---|---|---|---|---|---|
| BP + P (7:3) | 75 | 11 | 86 | 0.192 | 69.57 | 1.32 | 50.51 |
| BP + FP (7:3) | 71 | 13 | 84 | 0.212 | 43.36 | 1.59 | 46.10 |
| BP + CP (7:3) | 72 | 9 | 81 | 0.301 | 55.98 | 1.42 | 69.75 |
| BP + CTR + P (6:2:2) | 76 | 11 | 87 | 0.118 | 70.21 | 1.37 | 55.21 |
| BP + CTR + FP (6:2:2) | 72 | 13 | 85 | 0.208 | 63.34 | 1.67 | 57.84 |
| BP + CTR + CP (6:2:2) | 74 | 14 | 88 | 0.229 | 65.20 | 1.52 | 54.57 |
| BP + CTR + P (5:2:3) | 80 | 9 | 89 | 0.124 | 64.23 | 1.37 | 48.74 |
| BP + CTR + FP (5:2:3) | 74 | 10 | 84 | 0.300 | 49.72 | 1.59 | 51.56 |
| BP + CTR + CP (5:2:3) | 73 | 8 | 81 | 0.301 | 62.75 | 1.40 | 51.77 |
| BP + P + Z (7:2:1) | 76 | 10 | 86 | 0.204 | 62.40 | 1.37 | 35.10 |
| BP + P + Z (6:2:2) | 72 | 12 | 84 | 0.284 | 51.28 | 1.58 | 28.79 |
| BP + FP + Z (7:2:1) | 77 | 9 | 86 | 0.296 | 60.34 | 1.61 | 56.29 |
| BP + FP + Z (6:2:2) | 71 | 11 | 82 | 0.344 | 40.39 | 1.71 | 52.73 |
| BP + CP + Z (7:2:1) | 69 | 17 | 86 | 0.308 | 52.74 | 1.75 | 45.44 |
| BP + CP + Z (6:2:2) | 69 | 15 | 84 | 0.336 | 51.18 | 1.77 | 47.88 |
| BP + CTR + P + Z (5:2:2:1) | 74 | 13 | 87 | 0.328 | 46.67 | 1.63 | 38.82 |
| BP + CTR + FP + Z (5:2:2:1) | 67 | 13 | 80 | 0.296 | 40.00 | 1.68 | 41.52 |
| BP + CTR + CP + Z (5:2:2:1) | 71 | 13 | 84 | 0.376 | 61.35 | 1.54 | 43.74 |

*Seedling morphology*

Thirty seedlings from each treatment were destructively harvested in three replications after the second growing season and a variety of morphological traits were measured. The potting mix was carefully removed from the roots using both water and tweezers. The shoot height (SH) (cm) and root collar diameter (RCD) (mm) were measured and recorded. The shoots and roots were dried at 105°C for 24 hours and then weighed. Root dry weight (RDW) (g), shoot dry weight (SDW) (g) and shoot:root ratios were calculated. In addition, the dry root percentage (DRP) was obtained using RDW and the total seedling dry weight (TSDW).

*Experimental design and data analysis*

The experiment was arranged in a completely randomized block design with three replications for each treatment. A total of 18 treatments were randomly assigned to each block. Thirty seedlings per treatment were sampled at each sampling date.

The data were subjected to one-way analysis of variance (ANOVA). The variables were tested for normality and homogeneity of variances, and transformations were made when necessary to meet the underlying statistical assumptions of ANOVA. All pair-wise comparisons of individual means were done using the least significant difference (LSD) *t*-test at $P<0.05$. Relationships between growth medium properties and seedling morphological parameters were tested using correlation analysis.

*Table 4*
Initial chemical properties of growth media used in the study

| Growth medium symbols | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BP+P (7:3) | 5.30 | 57.65 | 0.80 | 1.270 | 23.1 | 1267 | 246 | 224 | 11200 | 18.34 | 71 | 0.20 | 9 | 68 |
| BP+FP (7:3) | 5.90 | 78.35 | 0.06 | 1.133 | 23.6 | 1013 | 106 | 169 | 85 | 5.32 | 70 | 0.10 | 6 | 24 |
| BP+CP (7:3) | 5.50 | 89.21 | 0.18 | 1.411 | 28.7 | 1797 | 172 | 237 | 194 | 5.44 | 101 | 0.0 | 7 | 42 |
| BP+CTR+P (6:2:2) | 5.50 | 102.26 | 0.35 | 1.773 | 18.1 | 2625 | 425 | 201 | 1098 | 63.61 | 62 | 0.0 | 11 | 127 |
| BP+CTR+FP (6:2:2) | 5.80 | 104.69 | 0.23 | 1.690 | 19.9 | 2898 | 461 | 201 | 1142 | 69.22 | 56 | 0.0 | 10 | 163 |
| BP+CTR+CP (6:2:2) | 5.60 | 99.82 | 0.23 | 1.679 | 18.9 | 2257 | 313 | 246 | 627 | 41.36 | 84 | 0.0 | 10 | 121 |
| BP+CTR+P (5:2:3) | 5.50 | 98.26 | 0.25 | 1.701 | 16.7 | 2169 | 397 | 203 | 1017 | 67.08 | 54 | 0.0 | 9 | 158 |
| BP+CTR+FP (5:2:3) | 5.80 | 96.26 | 0.22 | 1.803 | 16.6 | 2739 | 423 | 216 | 972 | 64.45 | 54 | 0.0 | 8 | 112 |
| BP+CTR+CP (5:2:3) | 5.40 | 126.78 | 0.46 | 2.007 | 15.0 | 3162 | 519 | 216 | 1188 | 66.91 | 71 | 0.2 | 11 | 155 |
| BP+P+Z (7:2:1) | 6.00 | 104.28 | 0.06 | 0.734 | 27.8 | 2134 | 149 | 562 | 4680 | 1.18 | 71 | 0.0 | 3 | 17 |
| BP+P+Z (6:2:2) | 5.80 | 96.52 | 0.05 | 0.587 | 28.5 | 4204 | 208 | 1221 | 10212 | 16.82 | 47 | 0.0 | 3 | 12 |
| BP+FP+Z (7:2:1) | 5.40 | 89.28 | 0.09 | 1.284 | 25.4 | 2242 | 164 | 533 | 3552 | 11.10 | 60 | 0.0 | 3 | 28 |
| BP+FP+Z (6:2:2) | 5.50 | 90.04 | 0.12 | 1.147 | 26.7 | 3893 | 204 | 1090 | 7412 | 3.04 | 60 | 0.0 | 3 | 21 |
| BP+CP+Z (7:2:1) | 5.60 | 103.30 | 0.14 | 1.157 | 22.8 | 3499 | 216 | 972 | 7560 | 8.28 | 70 | 0.0 | 4 | 24 |
| BP+CP+Z (6:2:2) | 5.80 | 98.60 | 0.10 | 1.091 | 25.5 | 3499 | 259 | 1188 | 9720 | 8.14 | 50 | 0.0 | 3 | 19 |
| BP+CTR+P+Z (5:2:2:1) | 5.70 | 103.76 | 0.18 | 1.352 | 16.7 | 3432 | 448 | 622 | 4884 | 54.67 | 48 | 0.0 | 7 | 79 |
| BP+CTR+FP+Z (5:2:2:1) | 5.40 | 105.65 | 0.48 | 1.758 | 13.7 | 4020 | 492 | 821 | 6912 | 74.19 | 31 | 0.0 | 7 | 144 |
| BP+CTR+CP+Z (5:2:2:1) | 5.70 | 120.65 | 0.48 | 2.068 | 12.2 | 4129 | 541 | 844 | 7992 | 72.79 | 46 | 0.0 | 7 | 88 |

1: pH (1 : 2.5 as volume); 2: CEC (me/100 g); 3: EC$\times 10^3$ mhos/cm; 4: Total N (%); 5: C/N; 6: $Ca^{++}$ ppm; 7: $Mg^{++}$ ppm; 8: $Na^{++}$ ppm; 9: $K^{+}$ ppm; 10: P ppm; 11: $Fe^{++}$ ppm; 12: $Cu^{++}$ ppm; 13: $Zn^{++}$ ppm; 14: $Mn^{++}$ ppm

## Results and discussion

*Seedling shoot height (SH) and root collar diameter (RCD)*

No significant differences were found between the treatments for SH and RCD ($P<0.001$) (Table 5). The general means of SH and RCD were 11.575 cm and 4.44 mm, respectively, in media with zeolite, and 12.193 cm and 4.6 mm in media without zeolite. The SH values found in this study were significantly lower than the values gives by Ayan (2002b) and Ayan and Bahadir (1995). The RCD values were close to the values observed by Ayan (2002b) and significantly higher than those found by Ayan and Bahadir (1995). The low SH values in this study can be explained by the inadequate air capacity (minimum 8–17%) in the growth media (Table 3). De Boodt and Verdonck (1972) stated that a growth medium providing perfect growing conditions must have both 20–25% of air volume and 20–30% of available water capacity at the same time. Aslan (1998) reported that the size of the aggregates in the growth medium significantly affected the root and shoot growth of seedlings. Similarly, the pore spaces in the growth medium could have been negatively influenced by the dust-size zeolite used in this study. This could be the reason why zeolite had no positive effect on SH. Similar findings were reported by Tuzuner and Timay (1984) and Koksaldi (1999).

*Table 5*
Mean values and multiple comparisons of morphology of 2-year-old seedlings

| Growth medium symbols | SH (cm) | RCD (mm) | Dry weight | | | |
|---|---|---|---|---|---|---|
| | | | SDW (g) | RDW (g) | SDW/RDW | DRP (%) |
| BP+P (7:3) | 13.104a | 4.982a | 3.198ab | 1.87a | 1.76a | 37.2a |
| BP+ FP (7:3) | 11.984a | 4.570a | 2.235abcd | 1.4abcdef | 1.602a | 38.6a |
| BP+CP (7:3) | 11.902a | 4.626a | 2.163abcd | 1.352abcdef | 1.582a | 38.8a |
| BP+CTR+P (6:2:2) | 12.773a | 4.676a | 2.221abcd | 1.307bcdef | 1.711a | 36.9a |
| BP+CTR+FP (6:2:2) | 11.536a | 4.437a | 2.307abcd | 1.541abcdef | 1.506a | 40a |
| BP+CTR+CP (6:2:2) | 11.808a | 4.333a | 1.935cd | 1.091ef | 1.792a | 36a |
| BP+CTR+P (5:2:3) | 10.498a | 4.343a | 2.097cd | 1.454abcdef | 1.453a | 41.1a |
| BP+CTR+FP (5:2:3) | 12.305a | 4.575a | 2.439abcd | 1.638abcd | 1.494a | 40.3a |
| BP+CTR+CP (5:2:3) | 13.831a | 4.890a | 2.875abc | 1.635abcde | 1.781a | 36.5a |
| Mean | 12.193 | 4.6 | 2.385 | 1.476 | 1.631 | 38.38 |
| BP+P+Z (7:2:1) | 12.394a | 4.386a | 2.458abcd | 1.416abcdef | 1.757a | 36.3a |
| BP+P+Z (6:2:2) | 9.367a | 4.180a | 1.593d | 1.145def | 1.385a | 42.4a |
| BP+FP+Z (7:2:1) | 11.217a | 4.303a | 1.941cd | 1.224cdef | 1.577a | 39.1a |
| BP+FP+Z (6:2:2) | 12.892a | 4.912a | 2.676abcd | 1.722abc | 1.543a | 40.2a |
| BP+CP+Z (7:2:1) | 13.053a | 4.737a | 2.124bcd | 1.197cdef | 1.722a | 37.6a |
| BP+CP+Z (6:2:2) | 9.958a | 3.655a | 1.788cd | 1.013f | 1.79a | 35.9a |
| BP+CTR+P+Z (5:2:2:1) | 10.617a | 4.453a | 1.788cd | 1.148def | 1.555a | 39.2a |
| BP+CTR+FP+Z (5:2:2:1) | 11.508a | 4.490a | 2.209abcd | 1.099def | 1.947a | 34.4a |
| BP+CTR+CP+Z (5:2:2:1) | 13.167a | 4.857a | 3.244a | 1.824ab | 1.811a | 36.4a |
| Mean | 11.575 | 4.44 | 2.202 | 1.310 | 1.676 | 37.94 |
| General Mean | 11.884 | 4.52 | 2.294 | 1.393 | 1.654 | 38.16 |
| F value | 1.677 ns | 0.918 ns | 2.010 * | 2.3514 ** | 0.843 ns | 0.812 ns |

Values are the means of three replications; for each character, mean values with the same letter are not significantly different at the 1% level; ***,**,*: significant at the $P < 0.001$, 0.01 and 0.05 levels, respectively; ns: non-significant

As shown in Table 6, there was no significant difference between media with or without zeolite in terms of SH and RCD. Thus, zeolite can be used instead of other additives (CP and FP). In the 3rd and 6th groups of media in which zeolite was compared with perlite, the addition of 10% zeolite had no effect on SH, but 20% zeolite had a negative effect. For RCD, both the 10% and 20% volume of zeolite gave results comparable to FP, CP and perlite (Table 6).

Guérin et al. (2001) found a relationship between the height of *Viburnum* and the physical parameters of the substrates: the tallest plants were obtained in substrates with the highest water availability and highest water content. No significant relationship could be established between height and the chemical parameters EC and CEC. Chong et al. (1994) used mushroom compost as a growth medium for *Weigela* culture and found that growth was a function of total pore space and not of chemical properties, as assessed by the EC value at the start of the experiment. In the present study, there was no correlation between soil chemical and physical properties and seedling characters (Table 7) except a positive correlation between EC and RCD and SH. A positive correlation was also found between the Cu content of the growth medium and

RCD. Ayan and Tüfeçioğlu (2006) found a negative correlation between Scots pine SH and K content. They also found that there was a significant positive correlation between RCD and the Mg and Mn contents of the growth medium.

*Seedling shoot and root dry weight (SDW, RDW)*

SDW ($P<0.05$) and RDW ($P<0.01$) differed significantly for the growth medium types. The highest SDW value (3.244 g) was observed in the BP + CTR + CP + Z (5:2:2:1) growth medium, while the lowest value (1.593 g) was observed in the BP + P + Z (6:2:2) growth medium. The maximum value of SDW was observed with a 10% volume of Z, while the 20% volume of Z reduced the SDW value significantly. The highest RDW values, determined in the BP + P (7:3) and BP + CTR + CP + Z (5:2:2:1) media, were 1.87 g and 1.824 g, respectively, and the lowest RDW value (1.013 g) in the BP + CP + Z (6:2:2) medium. The addition of 20% Z affected RDW negatively as affected SDW (Table 5).

*Table 6*

ANOVA results of treatments with added zeolite grouped with treatments without zeolite but the same medium types (n=18)

| Group | Growth medium symbols | Seedling morphology | | | | | |
|---|---|---|---|---|---|---|---|
| | | SH (cm) | RCD (mm) | Dry weight | | | |
| | | | | SDW (g) | RDW (g) | SDW/RDW | DRP |
| 1 | BP+CP (7:3) | 11.902 | 4.626 | 1.854 | 1.352 | 1.582 | 38.833 |
| | BP+CP+Z (7:2:1) | 13.053 | 4.737 | 1.772 | 1.197 | 1.722 | 37.600 |
| | BP+CP+Z (6:2:2) | 9.958 | 3.655 | 1.978 | 1.013 | 1.790 | 35.900 |
| | F value | 2.568ns | 1.244ns | 0.051ns | 0.741ns | 0.308ns | 0.256ns |
| 2 | BP+FP (7:3) | 11.984 | 4.570 | 1.872 | 1.400 | 1.602 | 38.633 |
| | BP+FP+Z (7:2:1) | 11.217 | 4.303 | 1.798 | 1.224 | 1.577 | 39.133 |
| | BP+FP+Z (6:2:2) | 12.892 | 4.912 | 2.570 | 1.722 | 1.543 | 40.167 |
| | F value | 0.501ns | 0.764ns | 0.666ns | 2.801ns | 0.026ns | 0.086ns |
| 3 | BP+P (7:3) | 13.104a | 4.982 | 3.015a | 1.870a | 1.760 | 37.200 |
| | BP+P+Z (7:2:1) | 12.394a | 4.386 | 2.162ab | 1.416ab | 1.757 | 36.333 |
| | BP+P+Z (6:2:2) | 9.367b | 4.180 | 1.346a | 1.145b | 1.385 | 42.400 |
| | F value | 6.778* | 1.940ns | 3.816* | 5.719* | 1.033ns | 1.106ns |
| 4 | BP+CTR+CP (6:2:2) | 11.808 | 4.333 | 1.935b | 1.091b | 1.792 | 36.000 |
| | BP+CTR+CP (5:2:3) | 13.831 | 4.890 | 2.874ab | 1.635a | 1.781 | 36.500 |
| | BP+CTR+CP+Z (5:2:2:1) | 13.167 | 4.857 | 3.244a | 1.824a | 1.811 | 36.400 |
| | F value | 2.189ns | 0.881ns | 5.141* | 10.763* | 0.005ns | 0.007ns |
| 5 | BP+CTR+FP (6:2:2) | 11.536 | 4.437 | 2.307 | 1.541a | 1.506 | 39.967a |
| | BP+CTR+FP (5:2:3) | 12.305 | 4.575 | 2.439 | 1.638a | 1.494 | 40.267a |
| | BP+CTR+FP+Z (5:2:2:1) | 11.508 | 4.490 | 2.209 | 1.099b | 1.947 | 34.367b |
| | F value | 0.140ns | 0.039ns | 0.120ns | 5.808* | 0.711ns | 8.718* |
| 6 | BP+CTR+P (6:2:2) | 12.773a | 4.676 | 2.221 | 1.307 | 1.712 | 36.933 |
| | BP+CTR+P (5:2:3) | 10.498b | 4.343 | 2.113 | 1.454 | 1.453 | 41.100 |
| | BP+CTR+P+Z (5:2:2:1) | 10.617b | 4.453 | 1.788 | 1.302 | 1.555 | 39.200 |
| | F value | 7.902* | 0.410ns | 0.505ns | 0.240ns | 2.167ns | 1.805ns |

Values are the means of three replications; for each character, mean values with the same letter are not significantly different at the 1% level; ***,**,*: significant at the $P < 0.001$, 0.01 and 0.05 levels, respectively; ns: non-significant

*Table7*
Pearson's correlation coefficients between growth medium properties and seedling morphological parameters (n=18)

| Properties | SH | RCD | RDW | SDW |
|---|---|---|---|---|
| pH | –0.303 | –0.439 | –0.159 | –0.271 |
| CEC | 0.109 | –0.150 | –0.119 | –0.08 |
| Organic matter | 0.315 | 0.217 | 0.202 | 0.158 |
| EC | 0.489* | 0.560* | 0.519* | 0.699* |
| N | 0.421 | 0.373 | 0.329 | 0.413 |
| C/N | –0.263 | –0.279 | –0.194 | –0.310 |
| Water-holding capacity | –0.051 | 0.012 | 0.202 | 0.005 |
| Air capacity | –0.150 | 0.245 | –0.419 | –0.268 |
| Porosity | –0.208 | –0.217 | –0.141 | –0.248 |
| Volume weight | 0.015 | 0.007 | –0.033 | 0.053 |
| Ignition loss | 0.256 | 0.122 | 0.262 | 0.280 |
| Specific gravity | –0.306 | –0.348 | –0.396 | –0.385 |
| Ca | –0.157 | –0.099 | –0.167 | –0.104 |
| Mg | 0.166 | 0.199 | 0.205 | 0.277 |
| Na | –0.327 | –0.309 | –0.317 | –0.239 |
| K | –0.139 | –0.061 | 0.11 | 0.130 |
| P | 0.100 | 0.173 | 0.178 | 0.198 |
| Fe | 0.369 | 0.247 | 0.102 | 0.097 |
| Cu | 0.467 | 0.475* | 0.484* | 0.565* |
| Zn | 0.345 | 0.373 | 0.295 | 0.312 |
| Mn | 0.167 | 0.191 | 0.184 | 0.201 |

*significant at $P<0.05$

Mixtures containing FP or CP with or without zeolite did not affect SDW and RDW. Moreover, in the 4th group of media, with CTR, 10% of zeolite added to CP showed a positive effect, but in media in which zeolite was used with perlite (Group 3), the addition of 20% zeolite reduced SDW and RDW (Table 6).

In this study, EC and Cu showed a significant relationship with the RCD, SDW and RDW of the seedlings, and EC also had a positive relationship with SH ($P<0.05$). The other soil properties did not show any significant correlation with the SH, RCD, SDW or RDW of the seedlings. Ayan and Tüfeçioğlu (2006) found a negative correlation between RDW and the Ca, Na and K contents of the growth medium in Scots pine seedlings, while RDW had a significantly positive correlation with Fe content and a negative correlation with the pH of the medium. There was also a negative correlation between the Na and K contents of the growth medium and SH in the same study.

*Dry shoot/root ratio (SDW/RDW) and dry root percentage (DRP)*

No significant differences were found in SDW/RDW or DRP between the treatments. The general means of the SDW/RDW ratio and DRP were 1.676 g and 37.94% for media with added zeolite, and 1.631 g and 38.38% for media without zeolite (Table 5). Thus, oriental spruce seedlings with high quality in terms of

DRP and SDW/RDW parameters could be propagated in media containing zeolite. In general, there were higher DRP values and more favourable SDW/RDW values in this study than in the study done by Ayan (2002b).

As shown in Table 5, there was no significant difference between growth media with and without zeolite in terms of the traits SH, RCD, SDW/RDW and DRP. Therefore, zeolite can be used as an additive material instead of CP, FP or perlite.

## Conclusions

The present study showed that zeolite could increase the SDW and RDW of containerised oriental spruce seedlings when it was mixed with growth media, and had a more positive effect on these attributes than FP, CP or perlite, though it reduced SDW and RDW when applied at a rate of 20% in combination with perlite. No significant differences were determined in terms of SH, RCD, SDW/RDW and DRP for potting media containing Z, FP, CP or perlite. In conclusion, as previously demonstrated for Scots pine seedlings (Ayan and Tüfeçioğlu, 2006), zeolite from Turkey can be used as an additive material in the propagation of containerized oriental spruce seedlings. Since Turkey has 45.8 billion tonnes of zeolite potential, using zeolite in container tree nurseries in Turkey may reduce the costs significantly. Furthermore, using zeolite offers a considerable advantage in respect of environmental pollution in forest nurseries.

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# RAPD VARIATION OF LATE-MATURING SORGHUM [*Sorghum bicolor* (L.) MOENCH] LANDRACES FROM ETHIOPIA

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A study on the extent and pattern of genetic variability in late-maturing sorghum [*Sorghum bicolor* (L.) Moench] landraces collected from the Wello and Hararge areas of Ethiopia was conducted using random amplified polymorphic DNA (RAPD) markers for 70 individuals representing 14 populations. Four oligonucleotide primers generated a total of 55 polymorphic bands with 13–19 bands per primer and a mean of 16 bands across the 70 individuals. The value of the Shannon diversity index among the populations (0.26) and between the two regions (0.24) was low to moderate, despite the high degree of polymorphic bands per primer. The mean genetic distance (0.25) between the populations was found to be low. The low genetic variation may be due to the reduced population size of late-maturing sorghum landraces in the two regions of Ethiopia because of farmers' decisions in the process of planting, managing, harvesting and processing their crops. Partitioning of the genetic variation into variation between and within the population revealed that 92.9% and 7.10% of the variation was found to be between and within the populations, respectively. Cluster analysis of genetic distance estimates further confirmed a low level of differentiation in late-maturing sorghum populations both between and within the regions. The implications of the results for genetic conservation purposes are discussed.

**Key words:** late-maturing, landrace, sorghum, genetic variation

## Introduction

Genetic variability is the raw material which gives species the opportunity to evolve under changing environments and selection pressures (Geleta, 2003). Knowledge about the amount and pattern of distribution of genetic variation in the germplasm collection is of great importance for designing future collection strategies and the management of genetic resources, as well as for the utilization of such resources in plant breeding programmes. Incorporating useful genes from crop landraces such as sorghum into breeding materials helps to increase

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yield and to maintain the stability of crop productivity by serving as a source for disease resistance and tolerance to insect pests as well as for tolerance to environmental stress, especially drought (Ayana, 2001; Gebru et al., 2002; Geleta, 2003). Molecular markers such as RAPD have the potential to identify a large number of polymorphisms with good coverage of the entire genome (Menkir et al., 1997). The RAPD technique has been used to characterize genetic diversity and phylogenetic relationships in sorghum (Menkir et al., 1997; Ayana et al., 2000).

The wide rage of environmental conditions under which sorghum is produced in Ethiopia has given rise to a tremendous range of genetic variability (Doggett, 1988), but the indigenous germplasm of sorghum has been threatened due to a series of adverse rainfall situations, forcing farmers to shift to short-season crops like teff (*Eragrostis tef*) and early-maturing uniform sorghum cultivars. This tendency is strengthened by the practice of relief agencies, who supply farmers with seeds of uniform varieties for rehabilitation purposes. In addition, farmers' decisions in the process of planting, managing, harvesting and processing their crops affect the genetic variability of the crop. There is thus a strong need to collect and conserve this invaluable genetic resource before it is lost for ever. According to Gebrekidan (1973, 1981) sorghum exists in tremendous diversity throughout the major growing areas in Ethiopia, which include pockets of isolation with an extremely broad and valuable, but vulnerable, genetic base for potential breeding and improvement. The present study used RAPD markers to determine the amount and pattern of distribution of genetic diversity in 14 late-maturing sorghum landraces from two geographical regions of Ethiopia.

## Materials and methods

*Plant material*

A total of 14 late-maturing sorghum landraces from the two regions of Ethiopia were obtained from the national sorghum research coordinating centre, Melkassa Agricultural Research Center. Seed from the selected landraces were germinated in pots in a greenhouse at Melkassa Agricultural Research Center. Fresh leaves of individual plants were harvested from 7- to 14-day-old seedlings for DNA extraction. The molecular work was done at Armauer Hanson Research Institute Laboratory, Addis Ababa, Ethiopia.

*RAPD analysis*

A total of 70 individual plants, five plants from each landrace, were used for DNA extraction (Table 1). Total genomic DNA was extracted from about 125 mg fresh leaves harvested from 7- to 14-day-old seedlings according to the method of Thomson and Henry (1993) with some modifications. Four primers (OPC-01, OPA-02, OPA-17 and OPA-18) giving strong reproducible bands were used.

The amplification reaction was performed in a final volume of 25 µl consisting of puReTaq Ready-to-Go PCR Beads and about 50 to 100 ng genomic DNA. Amplification was performed using a Hybaid Omnigene thermocycler with the simulated tube control function set at 25 µl. The amplification was programmed for 3 minutes at 95°C for initial strand separation

*Table 1*
Number and proportion of polymorphic loci and mean genetic diversity of the 14 sorghum genotypes in the population

| Accession number | No. of polymorphic loci | Percentage of polymorphic loci | Shannon index ± S.E. | Nei's genetic diversity ± S.E. |
|---|---|---|---|---|
| PGRC/E Acc # 69245 | 0.0 | 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| PGRC/E Acc # 69208-1 | 2.0 | 3.39 | 0.01 ± 0.01 | 0.01 ± 0.02 |
| PGRC/E Acc # 69228 | 1.0 | 1.69 | 0.01 ± 0.02 | 0.01 ± 0.02 |
| PGRC/E Acc # 228252 | 3.0 | 5.08 | 0.01 ± 0.02 | 0.02 ± 0.02 |
| PGRC/E Acc # 69185 | 4.0 | 6.78 | 0.02 ± 0.02 | 0.03 ± 0.03 |
| PGRC/E Acc # 69237 | 2.0 | 3.39 | 0.02 ± 0.02 | 0.02 ± 0.03 |
| PGRC/E Acc # 69241 | 4.0 | 6.78 | 0.02 ± 0.02 | 0.03 ± 0.03 |
| Wello Coll # 012-1 | 0.0 | 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| Harar Coll # 121 | 0.0 | 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| Harar Coll # 166 | 12.0 | 20.34 | 0.06 ± 0.03 | 0.09 ± 0.05 |
| Wello Coll # 038 | 3.0 | 5.08 | 0.01 ± 0.01 | 0.02 ± 0.02 |
| Wello Coll # 041 | 3.0 | 5.08 | 0.01 ± 0.02 | 0.02 ± 0.03 |
| Wello Coll # 072 | 7.0 | 11.86 | 0.05 ± 0.04 | 0.07 ± 0.05 |
| Local check (Rufie) | 3.36 | 5.69 | 0.02 ± 0.02 | 0.03 ± 0.03 |
| Mean ± S.E. | 3.17± 0.30 | 5.37± 0.31 | 0.26 ± 0.04 | 0.41 ± 0.06 |
| All populations | 55.0 | 93.22 | 0.26 ± 0.04 | 0.41 ± 0.06 |
| Region | | | | |
| Hararge | 46 | 77.97 | 0.26 ± 0.13 | 0.39 ± 0.18 |
| Wello | 41 | 69.49 | 0.22 ± 0.14 | 0.32 ± 0.20 |
| Mean ± S.E. | 44 ± 0.08 | 73.73 ± 0.32 | 0.24 ± 0.13 | 0.36 ±0.19 |
| All populations | 55 | 93.22 | 0.26 ± 0.12 | 0.41 ± 0.29 |

followed by 45 cycles of 1 minute at 94°C to denature the template DNA, 1 minute at 37°C to allow the primer to anneal to the target sequences and 2 minutes at the optimum temperature of 72°C, for DNA polymerase activity (chain elongation), using the fastest possible transition times between each temperature. The last cycle was followed by additional extension at 72°C for 10 minutes to ensure that the primer extension reaction was completed. Finally, the hold time was set to 4°C until samples were collected.

The amplification products were resolved by electrophoresis on 1.2% agarose gels run in 50 × TAE buffer, pH 8.0, for 2 hours at 100 V. The gel was stained with ethidium bromide (0.5 mg/ml), the DNA fragments (bands) were visualized under UV trans-illumination and images were taken with a computerized, programmed camera system. Following the assumptions of Lynch and Milligan (1994), each amplified RAPD band was treated as an independent character or locus and assigned a number in order of decreasing molecular weight. The size of each band was estimated using the 1 kb DNA molecular weight marker (100 base pair ladder). Each band was scored as present (1) or absent (0). Based on the recommendations of Lynch and Milligan (1994) only reliably scored bands were used for the analysis.

The magnitude of genetic variation was estimated using the Shannon-Weaver information measure described as:

$$H' = -\sum_{i=1}^{n} p_i \ln p_i$$

where $p_i$ is the proportion of amplified bands among the accessions in the $i^{th}$ region of origin and $n$ is the number of phenotypic classes for a character.

The proportion of variation attributed to differences between and within populations was determined using the formulas described by Nei (1975; 1976a):

$$Dst = H_t - H_s$$
$$Gst = (Ht - Hs)/Ht$$
$$Dm = Hs/Ht$$

where Dm = proportion of genetic variation within populations or regions; Gst = proportion of genetic variation between populations or regions; Hs = mean genetic variation of populations or regions; Dst = mean gene diversity between populations or regions; Ht = gene diversity in the total population or region. The pair-wise genetic distance between landraces and regions of origin was estimated using Nei's unbiased genetic distance (Nei, 1976b) based on the allele frequency data matrix. The genetic distances between populations and regions of origin were estimated using the formula:

$$D = - \ln [J_{xy}/ (J_x J_y)^{1/2}]$$

as suggested by Nei (1976b).

Based on the respective genetic distances, cluster analysis was performed using POPGENE (Yeh et al., 1997) software by the hierarchical method of clustering, known as the unweighted pair group method with arithmetic average (UPGMA) to estimate the relationships among the landraces. A dendrogram was constructed based on Nei's genetic distances.

## Results

The four primers generated a total of 55 RAPD bands, all of which were polymorphic across 70 individual samples from the fourteen landraces studied. A band (locus) was considered as polymorphic if it differentiated at least two of the 14 landraces. The number of amplification products per primer varied from 13 to 19 with a mean of 16. The size of the amplified DNA fragments ranged from 100 to 2200 base pairs. The RAPD band profile obtained with OPC-01 is shown in Figure 1. The proportion of polymorphic loci among the landraces varied from zero to 20.34 with an average of 5.37 (Table 1). Similarly, the percentage of polymorphic bands among regions varied from 77.97 for Hararge to 69.49 for Wello with a mean of 73.73. Almost all the amplified products were encountered at low frequency.

*Estimate of genetic diversity*

The extent of genetic variation for the 14 landraces was determined from the band frequencies using the Shannon-Weaver diversity index (Table 1). Pooled over the four primers, the mean Shannon information index varied from 0.32 for the Wello landraces to 0.39 for the Hararge landraces with a mean of 0.36. The estimate of genetic variation for the five regions of origin is given in Table 1. The mean for genetic variation varied from zero to 0.06 for the 14 landraces.

*Partitioning of genetic variation*

Genetic variation within the population accounted for 7.0%, while the remaining 93% was found between populations (Table 2). Similarly, 8.2% of the genetic variation was found within the regions of origin and the remaining 91.8% between the regions of origin of the landraces.

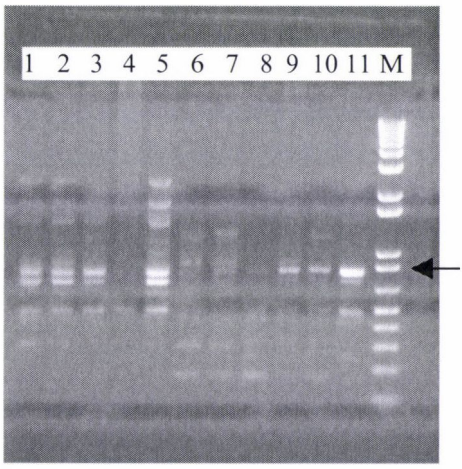



*Fig. 1.* Sample of RAPD (OPC-01) band pattern in eleven late-maturing Ethiopian sorghum [*Sorghum bicolor* (L.) Moench] landraces: 1. Wello Coll # 041-3, 2. Wello Coll # 041-4, 3. Wello Coll # 041-5, 4. PGRC/E Acc # 228252-1, 5. PGRC/E Acc # 228252-2, 6. PGRC/E Acc # 228252-3, 7. PGRC/E Acc # 228252-4, 8. PGRC/E Acc # 228252-5, 9. PGRC/E Acc # 69185-1, 10. PGRC/E Acc # 69185-2, 11. PGRC/E Acc # 69185-3, M: MW marker (1 kb DNA ladder). Arrow indicates 850 bp

The pair-wise genetic distances calculated between the 14 sorghum landraces based on Nei's genetic distance varied from 0.15 to 0.67 with a mean of 0.25. The highest pair-wise genetic distances were observed for PGRC/E Acc # 692418, followed by Harar Coll # 121, PGRC/E Acc # 69228 and PGRC/E Acc # 69245. The lowest pair-wise genetic distance (0.15) was obtained between PGRC/E Acc # 69208-1 and Wello Coll # 072.

As observed from the UPGMA dendrogram constructed using Nei's genetic distance (Nei, 1976b), there are three major clusters at Nei's genetic distance of 16.0, where the two major clusters further sub-clustered at varying genetic distances (Fig. 2). In the first cluster, there is only one genotype: PGRC/E Acc # 69241, which is from Hararge. This accession exhibited high genetic distances from Harar Coll # 121 (0.67), PGRC/E Acc # 69228 (0.54), PGRC/E Acc # 69245(0.51) and Acc # 69185 (0.51), which were higher than the mean genetic distance (0.25).

*Table 2*
Partitioning of the genetic diversity of sorghum genotypes into variation within and between populations and regions

| Region | Partitioning of Nei's genetic variation | | | | |
|---|---|---|---|---|---|
| | Ht | Hs | Gst | Dst | Dm |
| Hararge | 0.26 | 0.01 | 0.97 | 0.25 | 0.03 |
| Wello | 0.22 | 0.03 | 0.88 | 0.19 | 0.12 |
| Mean | 0.24 | 0.02 | 0.93 | 0.22 | 0.08 |
| All populations | 0.26 | 0.02 | 0.93 | 0.24 | 0.07 |

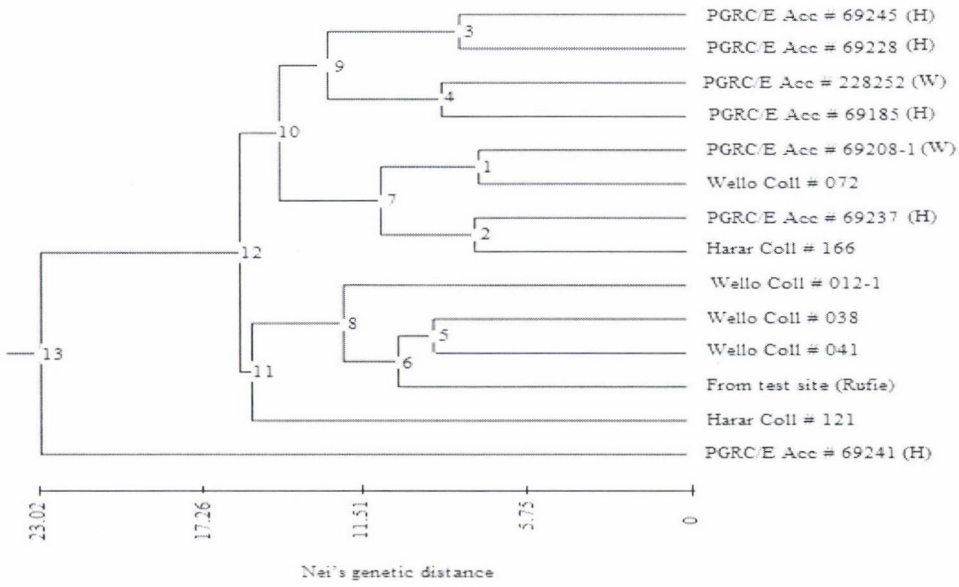



*Fig. 2.* Molecular dendrogram of fourteen Ethiopian sorghum landraces based on 55 RAPD fragments, amplified by four primers; the letters attached to the accessions represent the region of origin for each accession ('H' stands for Hararge and 'W' for Wello)

## Discussion

RAPD variation was analysed in 14 late-maturing sorghum landraces representing two regions of Ethiopia to determine the extent and distribution of genetic variation. The number of polymorphic bands generated per primer in the present study was high and is comparable with other previous studies for cultivated sorghum (Ayana et al., 2000). The high degree of RAPD polymorphic bands observed in the present study is appreciable compared with the highly reduced landrace population size in both the regions.

Contrary to the promising degree of RAPD band polymorphism, the amount of genetic variation, as estimated by the Shannon-Weaver diversity index, was found to be low to moderate when computed on the basis of both population and region of origin. In contrast to the work of Ayana et al. (2000) on wild sorghum, the partitioning of genetic variation indicated more variability between populations and regions than within populations and regions of origin. The genetic distance and identity recorded among genotypes from the RAPD data was so variable that genotypes with higher genetic distance and low genetic identity tended to come from different regions of origin. This implies that the relative differences are greater between regions of origin and between populations than within regions and populations. This suggests that sorghum genotypes should be sampled at a large number of sites to effectively represent the variation present between populations and between regions.

In conclusion, the data suggest that genetic distance estimates may help to identify suitable germplasm for introgression into breeding stocks. Selecting the most divergent landraces for introgression may increase the chance of extracting suitable inbred lines from backcross populations. Such inbred lines may, in turn, become useful sources of favourable alleles to improve the productivity of varieties and hybrids and their resistance to biotic and abiotic factors.

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# PRODUCTIVITY AND NITROGEN USE EFFICIENCY PARAMETERS IN COTTON CULTIVARS WITH VARYING N LEVELS AND SOIL TYPES UNDER RAINFED CONDITIONS

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A field experiment was conducted under rainfed conditions, on a shallow soil (Inceptisol) underlain with weathered basalt and on a deep soil (Vertisol) to evaluate three cotton cultivars [AKH 4 (*Gossypium arboreum*), LRK 516 (*G. hirsutum*) and NHH 44 (intra-*hirsutum* hybrid)] under four levels of N (0, 40, 80 and 120 kg $ha^{-1}$) and to analyse the variations in productivity using the nitrogen use efficiency (NUE) parameter. The yield of AKH 4 and NHH 44 was 101 and 89% higher than that of LRK 516. The yield and the response to N were higher on the Inceptisol. The enhanced yield and NUE of AKH 4 and NHH 44 were attributed to the improved efficiency of N uptake utilization. NUE declined from 21.6 at 40 kg $ha^{-1}$ to 7.7 at 120 kg N $ha^{-1}$. The N uptake efficiency and N utilization efficiency were independent of each other, but complemented each other in improving NUE. The implications of variations in NUE, N uptake efficiency and N utilization efficiency and their components, N biomass production efficiency and HI, in cotton breeding and agronomy are also discussed.

**Key words:** nutrient use efficiency, uptake efficiency, utilization efficiency, *Gossypium* spp., N response

## Introduction

Globally, rainfed cotton is cultivated on about 15.7 million ha, a third of which is in India. In many countries, diploid cottons (*Gossypium arboreum* and *G. herbaceum*) are being replaced by tetraploids (*G. hirsutums*) and the latter now account for 90% of global cotton production (Wendal and Cronn, 2003). However, the inherent ability of diploids to withstand biotic and abiotic stresses has renewed research interest in them (Deshpande et al., 2004).

Worldwide, cotton breeding is aimed at improving yield and fibre quality. Improved cultivars of *Gossypium* species respond positively to N application but the extent varies with the soil type and moisture availability. However, it is not understood whether this response is manifested through improved N uptake or N

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utilization or both. In maize, it was found to be due to a combination of high uptake and utilization efficiency, whereas in rape it was manifested through high uptake efficiency (Wiesler et al., 2001). Improvements in nitrogen use efficiency (NUE) are essential to sustain rainfed cotton production systems worldwide. The present investigation was therefore undertaken to compare the performance of one *G. hirsutum* and one *G. arboreum* cultivar along with an intra-*hirsutum* hybrid, under rainfed conditions, on two soils with varying N levels and to understand the response pattern in terms of N use efficiency parameters.

## Materials and methods

Field experiments were conducted on two soil types under rainfed conditions at the Central Institute for Cotton Research, Nagpur, India (21° 6' N and 79° 6' E), during the monsoon seasons of 2000–01 and 2001–02. In the 2000–01 crop season, 725 mm rainfall was received in 36 days, whereas in 2001–02, 893 mm rainfall was spread over 52 rainy days. The two soil types were a shallow (48 cm depth) well-drained Inceptisol underlain with a soft layer of weathered basalt, and a deep (150 cm depth) moderately well-drained Vertisol dominated by smectitic clay. The profile of the shallow soil had lower clay content (range 44–49%) than the deep soil (range 53–67%). Both the soils were non-saline but moderately alkaline (pH 7.8–8.1). The organic C content declined from 6 g $kg^{-1}$ in the surface layer to 1.8 g $kg^{-1}$ in the lowest (35–48 cm) horizon in the shallow soil and from 7.1 g $kg^{-1}$ in the surface layer to 3.0 g $kg^{-1}$ in the lowest (131–150 cm) horizon in the deep soil. The corresponding decline in available N (Subbiah and Asija, 1956) was from 91 to 28 mg $kg^{-1}$ in the shallow soil and 111 to 26 mg $kg^{-1}$ in the deep soil.

Three popular cultivars grown in the rainfed cotton belt were evaluated. The *G. arboreum* cultivar AKH 4 has medium staple in the fibre, is tall in stature and has medium growth duration. The *G. hirsutum* cultivar LRK 516 has long staple and is medium in stature and duration, whereas the intra-*hirsutum* hybrid NHH 44 has long staple, is medium in stature and has longer duration.

Two experiments were conducted, one each on shallow and deep soil. A split plot design with four replications was used with three cultivars (AKH 4, LRK 516 and NHH 44) in the main plots and four N levels (0, 40, 80 and 120 kg $ha^{-1}$) in the sub-plots. The crop was sown on 28 June 2000 and 6 July 2001 and raised with recommended practices. At maturity, five plants per plot were harvested, separated into leaves, stem, carpels, seed, lint and other floral parts, dried and weighed to calculate dry matter production. The N content in various plant parts was estimated (Piper, 1966) and total N uptake was calculated. The N content in the leaf litter was not taken into account. The seed cotton yield (lint + seed) was recorded for the entire net plot of 5.4 × 4.2 m. Data on the seed cotton yield, dry matter production and N uptake was subjected to ANOVA (Gomez and Gomez, 1984) separately each year for both soils. The apparent recovery (AR) was then computed using the following formula:

$$AR = \frac{\text{N uptake in } i^{th} \text{ treatment} - \text{N uptake in control}}{\text{N applied in } i^{th} \text{ treatment}} \times 100$$

Nitrogen use efficiency (NUE) and its components, N uptake efficiency (N Upt E) and N utilization efficiency (N Uti E), were calculated on a kg $kg^{-1}$ basis according to Moll et al. (1982), where: NUE = N Upt E × N Uti E and in turn N Upt E = Nt / Na and N Uti E = SCY / Nt, where Nt, Na and SCY denote N uptake, N applied and seed cotton yield, respectively. As proposed by Ortiz-Monasterio et al. (1997), N Uti E was further subdivided into harvest index (HI) and nitrogen biomass production efficiency (NBPE) and in turn NBPE = DMP/Nt, where DMP is dry matter production (at maturity).

## Results

### *Seed cotton yield and dry matter production*

The seed cotton yield of *G. arboreum* cultivar AKH 4 and intra-*hirsutum* hybrid NHH 44 was significantly higher than that of *G. hirsutum* cultivar LRK 516 in both years on either soil (Table 1). The mean increase over LRK 516 was 101% in AKH 4 and 89% in NHH 44. The mean increase in yield with AKH 4 over NHH 44 was 11.2%. The dry matter production followed a similar trend, the only exception being a non-significant difference between LRK 516 and NHH 44 on shallow soil in 2000–01. There was a significant response to N application in terms of yield on both soil types in 2000–01 and on shallow soil in 2001–02 (Table 1). On shallow soil, the incremental response was significant up to 80 kg $ha^{-1}$ in 2000–01 and up to 40 kg $ha^{-1}$ in 2001–02. The mean increase in yield over $N_0$ was 48.5% with 40 kg N $ha^{-1}$, 55.5% with 80 kg and 58.6% with 120 kg.

The cultivar × N interaction for seed cotton yield was significant on shallow soil in both years and on deep soil in 2001–02 (Table 1). On shallow soil in 2000–01, the N response of LRK 516 and NHH 44 was significant only up to 40 kg $ha^{-1}$, whereas AKH 4 responded to 80 kg N $ha^{-1}$. Unlike the other two cultivars, LRK 516 did not respond to N application on either soil in 2001–02.

*Table 1*

Effect of cultivars and N levels on seed cotton yield (kg $ha^{-1}$) and dry matter production (kg $ha^{-1}$) on shallow and deep soils

| N (kg) | Shallow soil | | | | Deep soil | | | |
|---|---|---|---|---|---|---|---|---|
| | AKH4 | LRK 516 | NHH 44 | Mean | AKH4 | LRK 516 | NHH 44 | Mean |
| | | | | Seed cotton yield 2000–01 | | | | |
| 0 | 661 | 570 | 649 | 627 | 641 | 409 | 931 | 661 |
| 40 | 1470 | 866 | 1322 | 1219 | 934 | 528 | 1199 | 886 |
| 80 | 2062 | 953 | 1428 | 1481 | 1023 | 627 | 1111 | 920 |
| 120 | 2261 | 941 | 1282 | 1494 | 1103 | 527 | 1079 | 903 |
| Mean | 1613 | 832 | 1170 | – | 925 | 523 | 1080 | – |
| S.E. Cultivar: 93.3, N: 102.4, Interaction: 108.6 | | | | | Cultivar: 56.8, N: 60.8, Interaction: 64.5 | | | |
| | | | | Seed cotton yield 2001–02 | | | | |
| 0 | 562 | 427 | 756 | 582 | 634 | 247 | 477 | 453 |
| 40 | 1024 | 417 | 1098 | 846 | 817 | 189 | 494 | 500 |
| 80 | 1034 | 467 | 934 | 812 | 600 | 230 | 388 | 406 |
| 120 | 1034 | 489 | 1150 | 891 | 518 | 256 | 424 | 399 |
| Mean | 913 | 450 | 985 | – | 642 | 230 | 445 | – |
| S.E. Cultivar: 47.2, N: 109.1, Interaction: 115.7 | | | | | Cultivar: 64.6, N: 35.5, Interaction: 37.6 | | | |
| | | | | Dry matter production 2000–01 | | | | |
| 0 | 2410 | 2207 | 2224 | 2281 | 1645 | 1171 | 1881 | 1565 |
| 40 | 4188 | 2534 | 3026 | 3249 | 2264 | 1649 | 2245 | 2053 |
| 80 | 5092 | 3962 | 3001 | 4018 | 2579 | 1687 | 2528 | 2265 |
| 120 | 5826 | 3595 | 3127 | 4183 | 2830 | 1693 | 2796 | 2440 |
| Mean | 4379 | 3075 | 2845 | – | 2330 | 1550 | 2363 | – |
| S.E. Cultivar: 189.4, N: 234.9, Interaction: 249.2 | | | | | Cultivar: 196.1, N: 153.2, Interaction: 162.5 | | | |
| | | | | Dry matter production 2001–02 | | | | |
| 0 | 2199 | 1058 | 2094 | 1783 | 2089 | 949 | 1786 | 1608 |
| 40 | 3816 | 1348 | 3342 | 2835 | 3013 | 835 | 1948 | 1932 |
| 80 | 3469 | 1231 | 2971 | 2557 | 2867 | 973 | 2129 | 1990 |
| 120 | 3245 | 1469 | 3861 | 2858 | 2911 | 961 | 2437 | 2103 |
| Mean | 3182 | 1277 | 3067 | – | 2720 | 930 | 2074 | – |
| S.E. Cultivar: 181.1, N: 185.3, Interaction: 196.5 | | | | | Cultivar: 102.9, N: 146.4, Interaction : 155.3 | | | |

The seed cotton yield was 55% higher on shallow soil than on deep soil (641 kg $ha^{-1}$). Similarly, the dry matter production was 49% higher on shallow soil than on deep soil. The mean yield in 2000–01 was 57% higher than in 2001–02.

*Nitrogen uptake*

On both shallow and deep soil, the average N uptake was highest in AKH 4, intermediate in NHH 44 and lowest in LRK 516 (Table 2). All the cultivars appeared to absorb more N from the shallow than the deep soil. In 2000–01, the N uptake increased with each 40 kg increment in applied N up to 80 kg N $ha^{-1}$, but the further increase at 120 kg N was not significant on either shallow or deep soil. However, in 2001–02, this increase was significant only up to 40 kg N $ha^{-1}$ on both soils.

*Apparent recovery of N and N use efficiency parameters*

Apparent recovery (AR) of N: Cultivars, N levels and soil types influenced the AR of N (Table 3). It ranged from 16–63% in AKH 4 (mean 37.7%), 10–69% in NHH 44 (mean 29.3%) and was the lowest (6–47%) in LRK 516 (mean 16.6%). In general, AR declined with increasing N levels. The mean AR values of N at $N_{40}$, $N_{80}$ and $N_{120}$ were 38.3, 25.0 and 20.3%, respectively. The average AR on shallow soil (35%) was higher than on deep soil (20%), and AR was higher (33%) in 2000–01 than in 2001–02 (23%).

NUE and its components: Irrespective of the soil type and cultivar, the NUE declined with an increase in N levels (Table 3). The mean decline was from 21.6 at 40 kg N to 11.3 at 80 kg N and further to 7.7 at 120 kg N $ha^{-1}$. The mean NUE of AKH 4 (17.2) and NHH 44 (15.3) was almost twice that of LRK 516 (8.1) and the NUE on shallow soil (16.7) was 60% higher than on deep soil. Wide variations (eightfold) ranging from 0.22 to 1.93 were observed in N Upt E and these variations paralleled the differences in NUE. Unlike N Upt E, the N Uti E varied across a narrower range between 8.7 and 22.6 and did not change greatly across N levels. The high-yielding cultivars AKH 4 and NHH 44 had higher N Uti E (17) than the low-yielding LRK 516 (13.7).

*Table 2*
Effect of cultivars and N levels on N uptake (kg $ha^{-1}$) on shallow and deep soils

| Cultivars | Shallow soil | | Deep soil | |
|---|---|---|---|---|
| | 2000–01 | 2001–02 | 2000–01 | 2001–02 |
| AKH 4 | 87.0 | 56.8 | 43.3 | 53.7 |
| LRK 516 | 62.5 | 25.8 | 29.9 | 23.7 |
| NHH 44 | 58.8 | 61.6 | 47.9 | 41.1 |
| S.E. | 4.51 | 2.75 | 3.70 | 2.00 |
| N (kg $ha^{-1}$) | | | | |
| 0 | 45.2 | 33.7 | 27.6 | 30.9 |
| 40 | 64.4 | 52.9 | 40.7 | 40.8 |
| 80 | 82.1 | 48.6 | 45.7 | 41.7 |
| 120 | 85.6 | 57.1 | 47.6 | 44.0 |
| S.E. | 4.91 | 4.62 | 2.84 | 3.23 |

*Table 3*
Apparent recovery (AR) of N, nitrogen use efficiency (NUE), N uptake efficiency (N Upt E) and N utilization efficiency (N Uti E) as affected by N levels and soil types

| | AR of N (%) | | NUE (kg kg$^{-1}$) | | N Upt E (kg kg$^{-1}$) | | N Uti E (kg kg$^{-1}$) | |
|---|---|---|---|---|---|---|---|---|
| | Shallow | Deep | Shallow | Deep | Shallow | Deep | Shallow | Deep |
| Year 2000–01 | | | | | AKH 4 | | | |
| $N_{40}$ | 62.0 | 36.0 | 36.8 | 23.4 | 1.93 | 1.09 | 19.1 | 21.4 |
| $N_{80}$ | 63.0 | 26.0 | 25.8 | 12.8 | 1.29 | 0.63 | 20.1 | 20.4 |
| $N_{120}$ | 53.0 | 18.0 | 18.8 | 9.2 | 0.97 | 0.42 | 19.5 | 21.8 |
| Year 2001–02 | | | | | | | | |
| $N_{40}$ | 60.0 | 55.0 | 25.6 | 20.4 | 1.62 | 1.52 | 15.8 | 13.4 |
| $N_{80}$ | 22.0 | 23.0 | 12.9 | 7.5 | 0.74 | 0.72 | 17.6 | 10.4 |
| $N_{120}$ | 18.0 | 16.0 | 8.6 | 4.3 | 0.52 | 0.48 | 16.4 | 9.0 |
| Year 2000–01 | | | | | LRK 516 | | | |
| $N_{40}$ | 25.0 | 23.0 | 21.7 | 13.2 | 1.33 | 0.76 | 16.3 | 17.3 |
| $N_{80}$ | 47.0 | 16.0 | 11.9 | 7.9 | 1.01 | 0.43 | 11.9 | 18.5 |
| $N_{120}$ | 25.0 | 11.0 | 7.8 | 4.4 | 0.61 | 0.28 | 12.8 | 15.4 |
| Year 2001–02 | | | | | | | | |
| $N_{40}$ | 16.0 | 7.0 | 10.4 | 4.7 | 0.66 | 0.56 | 15.8 | 8.4 |
| $N_{80}$ | 7.0 | 7.0 | 5.8 | 2.9 | 0.32 | 0.32 | 18.2 | 9.0 |
| $N_{120}$ | 9.0 | 6.0 | 4.1 | 2.1 | 0.26 | 0.22 | 15.7 | 9.6 |
| Year 2000–01 | | | | | NHH 44 | | | |
| $N_{40}$ | 55.0 | 39.0 | 33.1 | 30.0 | 1.58 | 1.2 | 20.9 | 25.0 |
| $N_{80}$ | 27.0 | 26.0 | 17.9 | 13.9 | 0.79 | 0.66 | 22.6 | 20.9 |
| $N_{120}$ | 22.0 | 22.0 | 10.7 | 9.0 | 0.56 | 0.49 | 19.0 | 18.5 |
| Year 2001–02 | | | | | | | | |
| $N_{40}$ | 69.0 | 12.0 | 27.5 | 12.4 | 1.69 | 0.98 | 16.2 | 12.7 |
| $N_{80}$ | 26.0 | 10.0 | 11.7 | 4.9 | 0.77 | 0.53 | 15.3 | 9.2 |
| $N_{120}$ | 31.0 | 12.0 | 9.6 | 3.5 | 0.65 | 0.41 | 14.8 | 8.7 |

Changes in the components of N Uti E, i.e. NBPE and HI, with N levels are depicted in Figure 1. Variations in NBPE were narrow, ranging from 35.9 in LRK 516 on deep soil with 120 kg N ha$^{-1}$ in 2001–02 to 59.1 in AKH 4 on shallow soil with 40 kg N ha$^{-1}$ in the same year (Table 3). No definite trend was observed across N levels or soil types. Among the cultivars the mean NBPE was slightly higher in NHH 44 (50.1) compared to LRK 516 (47.7) or AKH 4 (46.0). Moreover, in NHH 44, compared to the control ($N_0$) there was a consistent decline in NBPE with N applications. Unlike NBPE, the HI varied considerably (17.1 to 53.3%) for the different cultivars, N levels and growing conditions. The HI was higher in NHH 44 (35%) and AKH 4 (39%) than LRK 516 (30%) and higher on shallow soil (34%) than on deep soil (31%). In deep soil, barring $N_0$, there was a consistent decline in HI with increasing N rates in AKH 4 and NHH 44. On shallow soil, N application up to 80 kg ha$^{-1}$ increased the HI in AKH 4 in both years and in NHH 44 in 2000–01.

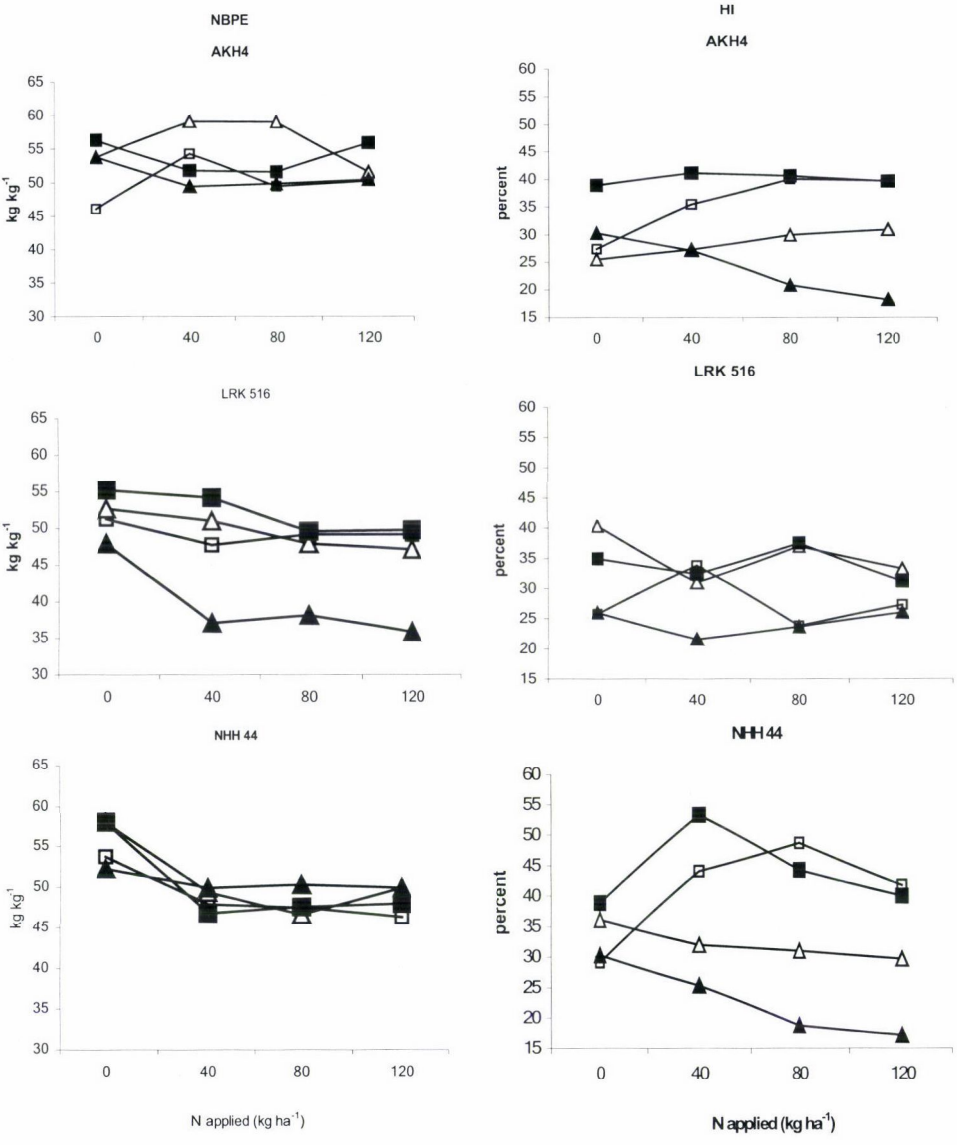



*Fig. 1.* Effect of N applications on nitrogen biomass production efficiency (NBPE) and harvest index (HI) in different cultivars. (□ shallow soil, year 2000–01, Δ shallow soil, year 2001–02, ■ deep soil, year 2000–01 and ▲ deep soil, year 2001–12)

There was a significant positive correlation between the AR of N and NUE (Table 4). The positive correlation between yield and NUE was either significant (5% level) or highly significant (1% level) in all cases except in AKH 4. The relationships between NUE and N Upt E and between N Uti E and HI were highly significant. The value of N Upt E was not correlated with N Uti E. At all N levels, the correlation between yield and N Upt E was highly significant and that between yield and N Uti E was significant.

*Table 4*
Simple linear correlation coefficients between yield, AR, NUE and its components as influenced by cultivars, N levels, soil types and year

| Parameter | Cultivar | | | N levels (kg ha$^{-1}$) | | | Soil type | | Year | |
|---|---|---|---|---|---|---|---|---|---|---|
| | AKH 4 | LRK 516 | NHH 44 | 40 | 80 | 120 | Shallow | Deep | 2000–01 | 2001–02 |
| Yield and AR | 0.60$^{*}$ | 0.83$^{**}$ | 0.59$^{*}$ | 0.84$^{**}$ | 0.86$^{**}$ | 0.94$^{**}$ | 0.66$^{**}$ | 0.68$^{**}$ | 0.73$^{**}$ | 0.73$^{**}$ |
| Yield and NUE | 0.51 | 0.65$^{*}$ | 0.56$^{*}$ | 1.00$^{**}$ | 1.00$^{**}$ | 1.00$^{**}$ | 0.53$^{*}$ | 0.70$^{**}$ | 0.49$^{*}$ | 0.71$^{**}$ |
| Yield and N Upt | 0.31 | 0.65$^{*}$ | 0.35 | 0.88$^{**}$ | 0.86$^{**}$ | 0.91$^{**}$ | 0.42 | 0.43 | 0.44 | 0.61$^{*}$ |
| Yield and N Uti | 0.60$^{*}$ | 0.37 | 0.88$^{**}$ | 0.73$^{*}$ | 0.67$^{*}$ | 0.70$^{*}$ | 0.55$^{*}$ | 0.89$^{**}$ | 0.36 | 0.67$^{**}$ |
| AR and NUE | 0.89$^{**}$ | 0.65$^{*}$ | 0.85$^{**}$ | 0.84$^{**}$ | 0.86$^{**}$ | 0.94$^{**}$ | 0.85$^{**}$ | 0.85$^{**}$ | 0.80$^{**}$ | 0.94$^{**}$ |
| NUE and N Upt | 0.93$^{**}$ | 0.94$^{**}$ | 0.91$^{**}$ | 0.88$^{**}$ | 0.86$^{**}$ | 0.91$^{**}$ | 0.96$^{**}$ | 0.86$^{**}$ | 0.95$^{**}$ | 0.97$^{**}$ |
| NUE and N Uti | 0.43 | 0.42 | 0.69$^{*}$ | 0.73$^{*}$ | 0.67$^{*}$ | 0.70$^{*}$ | 0.47$^{*}$ | 0.72$^{**}$ | 0.46 | 0.57$^{*}$ |
| N Upt and N Uti | 0.11 | 0.14 | 0.38 | 0.35 | 0.23 | 0.36 | 0.21 | 0.34 | 0.17 | 0.33 |
| N Uti and NBPE | 0.25 | 0.83$^{**}$ | –0.87$^{**}$ | 0.34 | 0.30 | 0.44 | –0.08 | 0.47$^{*}$ | –0.20 | 0.56$^{*}$ |
| N Uti and HI | 0.97$^{**}$ | 0.95$^{**}$ | 0.997$^{*}$ | 0.96$^{**}$ | 0.97$^{**}$ | 0.95$^{**}$ | 0.94$^{**}$ | 0.97$^{**}$ | 0.96$^{**}$ | 0.91$^{**}$ |

$^{*}$ and $^{**}$ indicate significance at the 5 and 1% level at n–2 d.f

## Discussion

The observed superiority of *G. arboreum* (AKH 4) and the hybrid (NHH 44) over *G. hirsutum* (LRK 516) was due to improved growth, higher N uptake, NUE and HI. *G. arboreum* AKH 4 was okra-leaved and the improved productivity might be attributed to lower stomatal conductance (Pettigrew et al., 1993), higher photosynthetic efficiency (Peng and Kreig, 1991) and improved HI (Venugopalan and Pundarikakshudu, 1999). This study also revealed a cultivar difference in N response on different soils, which was in agreement with the findings of Fritschi et al. (2003). The yield declined at 120 kg N ha$^{-1}$, presumably due to the altered source–sink relationship.

The improved productivity on shallow soil with a porous weathered basalt layer may be due to higher infiltration and better internal drainage. Conversely, the deep soil, which had a higher proportion of fine clay fraction, was poorly drained and the cotton plants experienced temporary water stagnation at different stages, retarding their growth. Hodgson and Chan (1982) reported that cotton was highly susceptible to excess water, so this could have reduced its productivity on deep soil. Mandal et al. (2005) also noted that the yield of rainfed cotton was higher on soils with better internal drainage. About 140 mm rainfall was received during October in the 2001–02 season, which altered the source–sink balance and reduced the yield compared to 2000–01.

A significant positive correlation between yield and the AR of N suggests that further yield improvements could be achieved in a sustainable manner through improvements in the AR of N. Further, variations in the AR of N paralleled variations in NUE, and hence the latter could be used as a parameter for breeding and agronomic manipulations. The values of NUE reported in this study were similar to those summarized by Gerik et al. (1998). The cultivars

differed in both NUE and in its components, N Upt E and N Uti E, at each level of N on both soils. There was a highly significant correlation between NUE and N Upt E. The higher yield and NUE of high-yielding cultivars AKH 4 and NHH 44 was due to higher N Upt E than in the low-yielding LRK 516 and to the more efficient utilization of absorbed N for yield formation. The variation in N Uti E among the cultivars was narrow. Nevertheless, in high-yielding cultivars, the correlation between N Uti E and yield was highly significant. Hence, in cultivars with an inherently high N Upt E, further yield improvements can be achieved by improving N Uti E. NBPE varied little across cultivars and was not sensitive to cultural manipulations (N application), soil type or rainfall variations and may not be a useful parameter. HI could be a more useful parameter for agronomic manipulations to achieve higher N Uti E.

The observed decline in NUE with increasing N levels was primarily due to a decline in N Upt E, indicating high loss of applied N. This was more evident on deep soils. A similar reduction in NUE at higher N levels was reported by Navarro et al. (1997). The highly significant correlation between yield and N Upt E indicates that to achieve high yields at high N rates, more efficient N delivery mechanisms are needed to reduce N losses, so that N Upt E could be increased.

The correlation between yield and NUE was higher in low-yielding environments (LRK 516, deep soil and poor rainfall distribution in 2001–02 compared to the high-yielding environment, (Table 4). However, the components of NUE, namely N Upt E and N Uti E, behaved independently and their contribution to yield under different situations complemented each other, with N Upt E being more important in low-yielding situations and N Uti E dominating in high-yielding environments. Thus, breeding or agronomic manipulations in low-yielding environments could aim at improving N Upt E, while if N Upt E is high further yield improvements may be possible by improving N Uti E.

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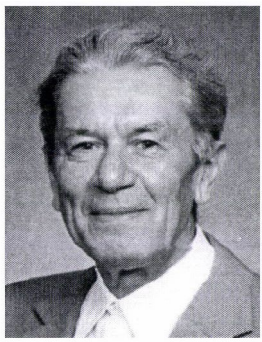
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# *Obituary*

## PROF. SÁNDOR RAJKI
## (1921–2007)



The fate of the Hungarian people during the twentieth century was hard and often cruel, forcing the nation to make superhuman efforts and sacrifices. It was against this background that Sándor Rajki spent his life exploiting his outstanding talents in the service of Hungarian agriculture.

The first 18 years of his life were spent on the small family farm in Pusztaföldvár, where he learnt the vital importance of land, wheat and bread, and the value of hard physical work. Heidegger wrote that the seed is the beginning and the end, the bearer of major inherited traits, the symbol of multiplication and spreading, of continuity and change, of survival, renewal and birth.

His parents, and the stark reality of life in south-eastern Hungary, taught Sándor Rajki to be firm and persevering. When his outstanding abilities became obvious, he was offered a scholarship to study in England, but his mother put her trust in the local teacher and declined to send her son abroad, despite the fact that finding money for school fees was no easy matter. In many cases, however, his school reports were so excellent that the school fees were waived, and he passed his final school examinations with distinction in Szarvas in 1939.

Over the following years he sought his vocation in many spheres, including studies at the University of Szeged, a clerical post at the Council offices in Pusztaföldvár, and a job as farm inspector in Northern Transylvania, which led to his enrolling at the Agricultural College in Kolozsvár. This proved to be a turning-point in his career, partly due to the influence of his excellent teachers, especially Alajos Mudra, professor of genetics, and partly to his fellow students, many of whom became lifelong friends and collaborators.

By the end of the war, in autumn 1945, he was working as an assistant lecturer at the University of Magyaróvár, after which he joined his friend Miklós

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Révy in the Education Department of the Ministry of Agriculture in 1948. A new period in his development as a scientist began in October 1949, when he travelled to the Soviet Union as a post-graduate student, with companions such as János Varga, György Pántos and Béla Győrffy, all of whom later played a decisive role in Hungarian agricultural research. He travelled widely and became well acquainted with the Soviet agricultural research programme of the time. One of his visits was to Krasnodar, where Lukyanenko had already developed Bezostaya 4, the predecessor of Bezostaya 1.

It was thus no coincidence that, when he returned to Hungary after obtaining his post-graduate degree and was appointed director of the Agricultural Research Institute in Martonvásár, he set up a project for the breeding of modern wheat varieties, based on 300 genotypes selected from the Vavilov gene collection in Leningrad, primarily consisting of varieties bred in the Soviet Union. The rationale for this was that it was becoming increasingly obvious in the mid-fifties that the Bánkúti wheat varieties, previously grown in Hungary and famous for their excellent quality and high protein content, were unsuitable for mechanical harvesting and for the higher mineral fertiliser rates required to obtain urgently needed increases in yield. In 1960 Sándor Rajki set himself the task of doubling wheat yields in Hungary, a target achieved within a few years due to his introduction of Bezostaya 1, followed later by other intensive wheat varieties.

Sándor Rajki was head of the Martonvásár institute from 1955 to 1980, a period of outstanding achievements by a team of dedicated scientists. Priority was given to two crops, maize and wheat, which were of major importance for Hungarian agriculture. It was at this time that the hybrid maize seed programme was initiated in Erdőhát, and maize hybrids from Martonvásár spread throughout the country.

A significant part of Rajki's work involved research on autumnisation, which both then and now induced very varied reactions within the scientific community. The book entitled "Autumnization and its Genetic Interpretation", published in 1967, drew the following comment from Prof. Agaev of Leningrad in a review in Botanicheskiy Zhurnal: "All in all the autumnisation research carried out by Rajki deserves the highest praise, as the data arose from experiments with a strictly applied methodology." This characterisation was a true reflection of Sándor Rajki, whose resolve to build a phytotron was fired by the need to create precise experimental conditions and reproducibility for research on autumnisation. This resolve reached fruition on 3 November 1972, when the first experiments were begun in the Martonvásár phytotron, one of Rajki's most lasting achievements. For the last 35 years, the phytotron has played a central role in research on plant genetics and physiology in Martonvásár, and continues to satisfy the criteria to be met for research on flowering biology, global climate change, stress resistance and gene

conservation in the 21st century. The staff of the Martonvásár institute frequently exploit the advantages of this research facility to further their participation in international scientific projects.

Retirement in 1982 did not mean a cessation of scientific activities but, thanks to his international reputation, invitations to work first for the Food and Agriculture Organisation in Rome, and later as a genetic consultant with the Garst company in the United States. This was not his first trip to America. Roswell Garst, the founder of the Pioneer Company, noted in his autobiography that when he visited Martonvásár in 1955, he was so impressed by Sándor Rajki that he insisted he should be a member of the official Hungarian delegation to the United States. It was as a result of this trip that the Hungarian authorities decided to build a hybrid maize seed plant in Martonvásár, an outstanding innovation, which was the first not only in Hungary, but also in the whole of Europe.

Sándor Rajki would have been unable to implement so many of his scientific visions without the help of his wife and closest colleague, Erna Ivanovna Cicer. Her capacity for hard work, and her perseverance and conscientiousness set a fine example to a whole generation of young scientists, to whom she taught the order and discipline that form the basis of research. Her integrity and encouragement were sadly missed in the Martonvásár phytotron when she retired.

A further pillar of strength was Imre Biacsi, the managing director of the Martonvásár institute under Sándor Rajki. Not only were they both born in Pusztaföldvár, but they studied together at the Agricultural Academy in Kolozsvár. During his years as managing director, Imre Biacsi spared no effort to take the load of administrative duties off the shoulders of Sándor Rajki. For more than two decades they formed an ideal team, in which Sándor Rajki was the scientific strategist, while Imre Biacsi managed everything from the day-to-day running of the seed plant to ensuring that there was a continuous supply of sunflower seeds for the bird-table in front of the phytotron.

There was a similarly close relationship between Sándor Rajki and András Kuti, who contributed to the success of the Martonvásár seed business as head of the Experimental Farm in Erdőhát. When trumped-up charges were made against him by the state and party authorities, Sándor Rajki did not desert him, but sat behind him every time he appeared in court. This experience gave him an insight into the demagogy and lies that lay at the heart of the party-state, and played no small part in his decision, in 1979, to resign from the Communist Party, a step that very few people dared to take at that time. It was a difficult decision, not because he was afraid of the consequences, which – as he foresaw – destroyed his scientific career, but because he had joined the party with youthful ardour, believing in the ideals of a brave new world.

Sándor Rajki had a frank, open personality, never recoiling from the cut and thrust of debate. One form taken by his outspokenness was the establishment of the Széchenyi Club in Martonvásár, in which he played an active part until it

was banned by the authorities. The club members did not take this lying down, but established the Brunszvik Club, which continued the tradition of the Széchenyi Club right up to the change of regime.

After spending much of the 1980s abroad, Sándor Rajki was elected as a full member of the Hungarian Academy of Sciences in 1991. In his inaugural speech he spoke exclusively about his work in Rome and the United States, thus wisely conceding his detachment from current Hungarian scientific life. He spent his remaining years quietly with his family in Gödöllő, far from the commotion of public life after the change of regime. If the conversation sometimes turned to politics, he would just smile to himself and say little. Many of us could learn from him how to retire gracefully, with head held high. As a result, he will be remembered as he was in his prime, for his great intellect and lasting achievements. Sándor Rajki can truly be said to have fulfilled his vocation.

My generation became acquainted with Sándor Rajki when we started working in Martonvásár in the seventies. He had a good insight into character, and was expert at choosing the right man for the right job. He constantly encouraged us to ignore fashionable research topics, where it was easy to make headway, and to give our attention to problems that appeared to be insoluble and represented a real challenge. We can now say with Newton: "If I have seen farther than others, it is because I was standing on the shoulders of giants." For us, Sándor Rajki was one of the giants.

It is difficult to speak of Sándor Rajki's personality, because those who never knew him tend to have a false picture of him. The multifaceted nature of his character made him at once a uniquely colourful personality and unfathomable. His strong, firm principles and his wide-ranging activities hallmarked a period in Martonvásár when Hungarian agriculture was in the ascendant. His achievements will survive him: the phytotron, the Martonvásár wheat varieties and the research community he established in the Martonvásár institute all represent a continuation of his efforts.

In the 4$^{th}$ chapter of the Gospel according to St. Mark we read: "And other fell on good ground, and did yield fruit that sprang up and increased; and brought forth, some thirty, and some sixty, and some an hundred." It is a measure of Sándor Rajki's greatness that he achieved so much despite the contradictions and struggles of the times in which he lived.

Z. BEDŐ

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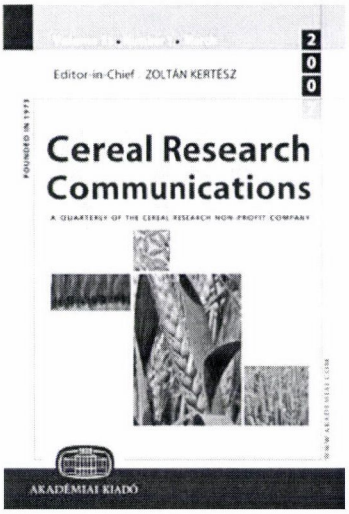
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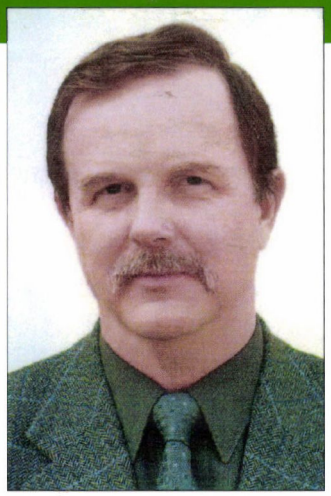
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# CONTENTS

ORIGINAL PAPERS

Effect of combinations of light intensity and photoperiod on heading date of barley (*Hordeum vulgare* L.)
*I. Karsai, K. Mészáros, B. Kőszegi, Z. Bedő* and *O. Veisz* ..... 397

Comparison of germination responses of cultivated wheat (*Triticum*) and its wild relative (*Aegilops*) species under salinity, temperature and light
*M. Yildiz* and *E. Kasap* ..... 407

Study of the effect of plant density on the growth of maize (*Zea mays* L.) hybrids using the Richards function
*Z. Berzsenyi* and *Q. L. Dang* ..... 417

Sulphur management in moong (*Phaseolus aureus* L.) and raya (*Brassica juncea* L.) crops
*M. P. S. Khurana* and *R. L. Bansal* ..... 437

Correlation between maize genotypes and the stalk rot caused by maize *Fusarium*
*C. Szőke, T. Árendás, F. Rácz, J. Pintér, E. Nagy* and *C. L. Marton* ..... 447

Soil changes after forty years of succession in an abandoned coppice in the Czech Republic
*R. Hédl* and *K. Rejšek* ..... 453

Standardization of dosage of liquid and cyst formulations of *Azospirillum* for different application methods
*R. Thamizh Vendan* and *M. Thangaraju* ..... 475

SHORT COMMUNICATION

Influence of oil content on the equilibrium moisture content of evening primrose (*Oenothera* spp.) seeds
*A. F. Fieldsend* ..... 485

REVIEW

Role of S-methylmethionine in the plant metabolism
*D. Szegő, E. Kósa* and *E. Horváth* ..... 491

LIST OF REVIEWERS OF VOLUME 55, 2007 ..... 509

INDEX OF VOLUME 55 ..... 511

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# EFFECT OF COMBINATIONS OF LIGHT INTENSITY AND PHOTOPERIOD ON HEADING DATE OF BARLEY (*Hordeum vulgare* L.)

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In order to evaluate the effect of light intensity and photoperiod on heading and to establish the reaction types of barley, a set of barley germplasm of various geographical origin and growth habit was examined in a series of controlled growth chamber experiments combining two levels of light intensity with long and short photoperiod regimes. Low light intensity contributed only a limited portion to the total variance of heading and this originated to a large extent from the genotype × light intensity interaction for both photoperiods. Under the long photoperiod regime the effect of low light intensity was only apparent in a significant delay in heading. Under a short photoperiod the type of sensitivity depended on the growth habit. Low light intensity hastened plant development in 15% of the spring barley varieties, while the flowering of 44% of the winter barley varieties was significantly delayed. Establishing the reaction types for photoperiod and low light intensity in this range of barley germplasm made it possible to identify the typical reaction types of the two growth-habit groups. In addition, it also became possible to identify genotypes with contrasting or unusual combinations of these traits.

**Key words**: light intensity, photoperiod, flowering, *Hordeum vulgare* L.

## Introduction

In addition to supplying the energy source for photosynthesis, light has a significant effect on plant development and flowering. The duration (photoperiod), quality (spectral composition) and quantity (intensity) of the light all participate in the genetic determination of flowering as input signals at several levels of the complex controlling mechanisms (Imaizumi and Kay, 2006; Zhou et al., 2007). Of these characteristics, the effect of photoperiod has been studied to the largest extent, due to its major importance. While all the other environmental factors may show tremendous variation from year to year, in temperate zones changes in the photoperiod follow the same yearly rhythm in

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synchrony with the seasons. Thus, the photoperiod-based control of flowering has evolved in temperate plant species, and plays a basic role in anticipating and preparing for the expected environmental changes. The central element of photoperiod-based control is the circadian rhythm, the inner timekeeper of plants. This central oscillator, entrained by fluctuating environmental factors, regulates daily fluctuations in the activities of various genes involved in the photoperiod developmental pathway, thus ensuring phase-specific responses (McClung, 2006; Zhou et al., 2007). The basic photoperiod response thus achieved may be further modulated by the spectral composition of light. The ratio of far-red and red light is decisive for flowering (Deitzer et al., 1982). While far-red (>700 nm) and blue light (400–500 nm) have an enhancing effect, red light (600–700 nm) often delays flowering (Lin, 2000). Various small families of photoreceptors – phytochromes, cryptochromes and phototropins – have evolved in plants for sensing special spectral components. As environmental cue sensors, the photoreceptors provide input signals to the central oscillator, which in turn gates their sensitivity to the light, an interacting process in which light intensity also has a significant role (Gardner et al., 2006; Hotta et al., 2007). Recent results in *Arabidopsis* revealed that the photoreceptors take part not only in light spectrum perception but also in sensing light intensity, and their effects are also influenced by the temperature, thus underlining the complex interactive roles of various environmental factors (Zhou et al., 2007). Increasing light intensity has an accelerating effect on the period of the circadian rhythm, which is thought to participate in the continuous adjustment of the period of the central oscillation throughout the light phase (Gardner et al., 2006). The association between light intensity and flowering, however, is not linear, as low light intensities combined with a change in the far-red/red ratio were found to participate in the shade avoidance mechanisms of plants living in natural habitats, resulting in both earlier flowering and elongation (Vandenbussche et al., 2005).

The effect of photoperiod on the flowering of cereals has been studied in depth, establishing not only the various reaction types but also the major genes regulating photoperiod sensitivity and their influence on heading (Laurie et al., 1994; Karsai et al., 1997; Turner et al., 2005; Faure et al., 2007). In general, barley is a facultative long day plant; its heading is enhanced by a long photoperiod, but it is able to head under short photoperiods as well (Karsai et al., 2001; Laurie et al., 2004). There is, however, great variation in the photoperiod sensitivity of barley genotypes, which is apparent both in the enhancing effect of long photoperiods and in the delaying effect of short photoperiods (Karsai et al., 2001). Much less is known on the role and effect of light intensity in determining the heading date in cereals. In spring wheat, Evtushenko and Chekurov (2004) examined the effect of low light intensity on heading and found it to be mostly independent of photoperiod sensitivity and under the genetic control of a few recessive genes. They concluded that these recessive

genes may coincide with the *eps* loci and that sensitivity to low light intensity may contribute a large portion of the heading date variance between wheat cultivars of different geographical origin. When studying the reactions of two barley varieties, the parental lines of a mapping population, light intensity was found to have a significant effect on heading as a function of temperature, but even in this limited sample there was a substantial difference between the genotypes, especially at temperatures higher than 15°C and at light intensities lower than 340 μmol $m^{-2}$ $s^{-1}$ (Karsai et al., 2008).

The aim of the present work was to study the effect of two major characteristics of light, low light intensity and photoperiod, on heading and to establish the various reaction types in a set of barley germplasm.

## Materials and methods

Descriptions of the 38 barley varieties (*Hordeum vulgare* L.) involved in the present experiments were detailed in Karsai et al. (2001). There were 23 of North American, 7 of West European and 8 of East European origin. Based on their growth type there were 21 spring, 15 winter and 2 facultative barley varieties.

*Phytotron experiments*

The effect of two light intensities – 170 μmol $m^{-2}$ $s^{-1}$ (low intensity) and 220 μmol $m^{-2}$ $s^{-1}$ (medium intensity) – in combination with various photoperiod regimes on the heading date of the 38 barley varieties was studied in two separate experiments. The medium light intensity (220 μmol $m^{-2}$ $s^{-1}$) was applied at photoperiods of 10 h (short day), 14 h and 18 h (long day), while the low light intensity (170 μmol $m^{-2}$ $s^{-1}$) was applied at photoperiods of 10 h (short day), 12 h and 16 h (long day). Each experiment was carried out with the same experimental design in CONVIRON (Conviron Ltd., Winnipeg, Canada) growth chambers (Karsai et al., 2001; 2005). The technical parameters of the growth chambers (source of light; temperature and light intensity controls) are fully detailed in Karsai et al. (2004). In all cases the seedlings were vernalized for six weeks at 3°C with a 9 h light/15 h dark photoperiod regime and minimum light intensity (10 μmol $m^{-2}$ $s^{-1}$), after which the plants were planted individually in pots 12 cm in diameter and 18 cm in height, with a soil capacity of 1.5 kg. Each variety was replicated twice within the treatments, giving an average plant density of approximately 60 plants/$m^2$. In both experiments the temperature was kept constant at 18°C day and night throughout the growing period. Watering was carried out daily and fertilisers were applied regularly, twice a week at the beginning of plant development and every other day later. The heading date was evaluated by recording developmental phase 49 (Dev49), based on the scale of Tottman and Makepeace (1979). The experiments were terminated 200 days after planting and plants that had not headed by this time were given a Dev49 value of 200 for statistical purposes.

As one-way ANOVA did not show significant differences in the heading dates under the 18 and 16 h photoperiods (P=0.111), the factorial ANOVA module of the Statistica 6.1 program was applied to evaluate the variance components of heading date at the two light intensities under long and short photoperiod regimes. Based on the heading dates under the six different environments, the varieties were grouped by applying the hierarchical cluster analysis module of the Statistica 6.1 program.

## Results

*Effect of light intensity and photoperiod on heading date*

In analysing the variance components of heading date, the single factor with the highest contribution was photoperiod (SS%=55.8%), followed by the genotype (SS%=23.8%), while light intensity made a very limited contribution both alone (SS%=0.05%) and through its interactions with the other components (the lowest SS%=0.07% was for photoperiod × light intensity, and the highest SS%=1.9% for photoperiod × light intensity × genotype). When the analyses were partitioned into long or short photoperiods, light intensity again contributed only a low portion of the variance. Under long photoperiod this value was 7.2% and originated to a similar extent from the light intensity and the light intensity × genotype interaction. Under short photoperiod, however, the contribution of 9.0% SS was derived solely from the light intensity × genotype interaction.

Under the long photoperiod regime, the average heading date was 42 days for the 18 h daylength with medium light intensity, and 47 days for the 16 h daylength with low light intensity (r= 0.93, significant at the P=0.001 level) (Fig. 1a). The variation in heading was larger for the winter barleys than for the spring barleys. At 18 h and medium light intensity the heading date interval was between 26 and 71 days for all the varieties, with a range of 26–38 days for spring varieties and 28–71 days for winter varieties. At 16 h and low light intensity these data were the following: 32–75 days for all the varieties, 32–46.5 days for spring genotypes and 31.5–75 days for winter barleys (Fig. 1a). Under the long photoperiod regime the effect of low light intensity was only apparent in a significant delay in heading ($LSD_{P=0.05}$ = 5.9 days between the two light intensities); spring barleys proved to be more sensitive to this treatment, as 10 of the 21 genotypes (48%) headed later under low light intensity, while in the case of winter barleys this was true of only four out of 15 (27%).

Under a short photoperiod (10 h) the average heading date was 118 days in both experiments, though there were considerable variations in the reaction type of the individual varieties, caused by changes in light intensity (r=0.84, significant at the P=0.001 level) (Fig. 1b). At medium light intensity the heading date interval of the whole germplasm was 72–200 days with five varieties not able to head, while at low light intensity it was 59.5–200 days with seven varieties not able to head. Under a short photoperiod low light intensity significantly influenced the heading of 22 varieties ($LSD_{P=0.05}$ = 13.0 days between the two light intensities), but in this case not only the delaying but also the enhancing effect of low light intensity was apparent. The enhancing effect was more characteristic of spring genotypes, as ten of the 13 varieties that headed earlier under low light conditions had spring growth type (48% of all the spring varieties), while three were of winter type (20% of all the winter barleys). The delaying effect of low light intensity, on the other hand, was more characteristic of winter barleys, as eight of the nine varieties with significantly later heading under low light intensity belonged to the winter barley group (53% of all the winter barleys) and only one to the spring group (5% of all the spring barleys).

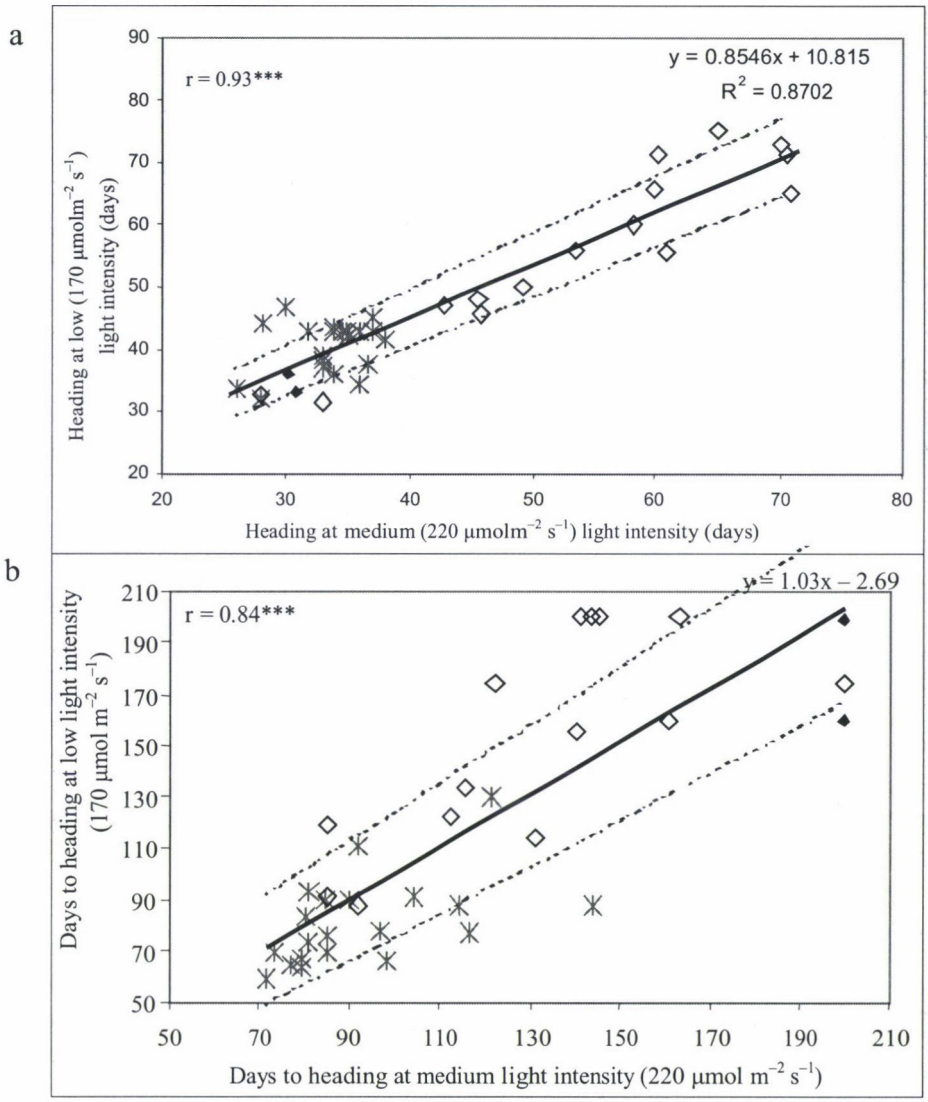



*Fig. 1.* Effect of two light intensity levels on the heading date characteristics of 38 barley varieties in controlled environmental chambers with (a) long photoperiod or (b) short photoperiod regimes at a constant temperature of 18°C. (∗) spring, (♦) facultative and (◊) winter barley varieties

*Genotypic variation in sensitivity to light intensity and photoperiod*

To obtain a better assessment of the reaction types of barley varieties, hierarchical cluster analyses were carried out on the combined data matrices of the six heading date sets from the two independent experiments. The 38 varieties could be separated into four major groups with significantly different heading date characteristics (Fig. 2, Table 1). With respect to the growth habits of the varieties, Groups 1 and 3 represented uniform groupings, as the 14 varieties

belonging to Group 1 were all of spring growth habit, while those in Group 3 were of winter growth habit. Group 2 consisted of five spring and seven winter barleys, while in Group 4 there were two winter, two facultative and one spring barley, all of US origin. The other three groups included varieties of various geographical origin (Fig. 2).

*Table 1*

Average heading dates of the four variety groups based on Joining tree clustering and confirmed by K-means clustering under various combinations of photoperiods and light intensities

| Groups | No. of genotypes in the group | Days to heading for various combinations of photoperiod (hours) and light intensity (μmol $m^{-2}$ $s^{-1}$) | | | | | | P value for treatments |
|---|---|---|---|---|---|---|---|---|
| | | 18 + 220 | 16 + 170 | 14 + 220 | 12 + 170 | 10 + 220 | 10 + 170 | |
| Group 1 | 14 | 33 a\a | 40 a\b | 47 a\c | 64 a\d | 86 a\f | 75 a\e | 0.000 |
| Group 2 | 12 | 44 b\a | 48 b\a | 61 b\b | 75 b\c | 109 b\d | 107 b\d | 0.000 |
| Group 3 | 7 | 58 c\a | 59 c\a | 66 b\a | 93 c\b | 145 c\c | 184 c\d | 0.000 |
| Group 4 | 5 | 38 ab\a | 44 ab\ab | 58 b\bc | 93 c\c | 200 d\d | 187 c\d | 0.000 |
| P value for groups | | 0.000 | 0.005 | 0.000 | 0.000 | 0.000 | 0.000 | |

Values followed by the same letter are not significantly different from each other at the P=0.05 level; between groups\between treatments

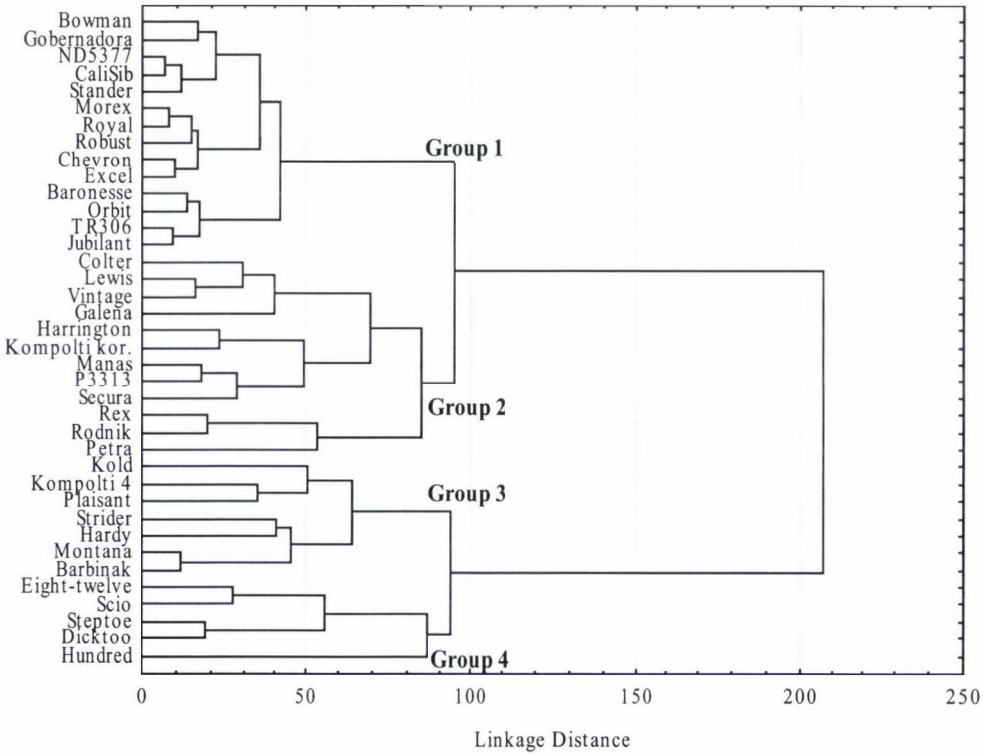



*Fig. 2.* Dendrogram of the 38 barley varieties based on their heading date characteristics under six environments combining various photoperiods and light intensities

The spring barley varieties in Group 1 were the earliest to head in all the environments, showing a medium level of photoperiod sensitivity (Table 1). Light intensity significantly influenced the average heading in a photoperiod-dependent way; under a long photoperiod lower light intensity significantly delayed flowering, while under a short photoperiod it had a significant enhancing effect. The delaying effect detected at long photoperiod was the most pronounced in the subgroup consisting of Baronesse (D), Orbit (Slo), TR306 (Can) and Jubilant (Slo), and the enhancing effect under a short photoperiod in the subgroup Bowman, Gobernadora, ND5377, CaliSib and Stander (all of US origin). The winter barleys in Group 3 were among the latest to head in all the treatments. The photoperiod sensitivity of this group was confined to the short photoperiod regimes, as there was no significant difference in heading between the 14 and 18 h daylight treatments. Light intensity had no great effect on this group under a long photoperiod, while it significantly delayed heading under a short photoperiod, also resulting in greater photoperiod sensitivity. This delaying effect was the strongest for the subgroup Strider (USA), Hardy (A), Montana (USA) and Barbinak (Ukr), which were not able to head at all in the lower light intensity treatment. The heading date characteristics of Group 2, containing both spring and winter barleys, were intermediate to those of Groups 1 and 3; on average, light intensity had no significant effect on heading under either photoperiod. Within this group, the subgroup Colter (USA), Lewis (USA), Vintage (UK) and Galena (USA) represented a special type, as they headed as late as the other winter barley members of the group when a short photoperiod was combined with medium light intensity, but were more responsive to low light intensity. Low light intensity significantly delayed the heading of another subgroup, Harrington (Can), Kompolti Korai (H) and Manas (Ukr), which nevertheless headed significantly earlier than the genotypes in Groups 3 and 4. The varieties in Group 4 showed the greatest photoperiod sensitivity among the barleys studied. Under a long photoperiod they were among the earliest to head, while under a short photoperiod they were among the latest. On average, low light intensity delayed their heading under a long photoperiod regime, and this delay was significant in the case of Hundred (USA) and Steptoe (USA). Under a short photoperiod the light intensity influenced the heading of the subgroup Eight-twelve (USA) and Scio (USA), which were only able to head under low light and not at medium light intensity, while the subgroup Steptoe (USA), Dicktoo (USA) and Hundred (USA) was not able to head at all under a short photoperiod regime, irrespective of the light intensity applied.

## Discussion

Controlled environment tests represent a unique opportunity to dissect complex environmental stimuli into individual factors, and to study the effects of these individual factors on plant development. Similar to the comparison of the effects of temperature and light intensity on heading (Karsai et al., 2008), the present results indicate that light intensity influences heading date primarily

through its association with photoperiod in a strongly genotype-dependent way. Under a long photoperiod the significant effect of low light intensity was associated with delayed flowering in every case. On the other hand, low light intensity showed a dual effect under short photoperiod; one-third of the barley germplasms studied were sensitive to low light intensity, though the type of sensitivity depended on the growth habit. Low light intensity hastened plant development in 15% of the spring barley varieties, while the flowering of 44% of the winter barley varieties was significantly delayed. The association between the shade avoidance mechanism of *Arabidopsis* and the sensitivity to low light intensity in cereals is not yet clear-cut, but the reaction type of spring barley varieties sensitive to low light intensity shows strong similarities (Vandenbussche et al., 2005). These findings may indicate that in cereals light intensity plays a chaperon role to photoperiod and temperature in entraining the circadian rhythm, as in the case of *Arabidopsis* (Zhou et al., 2007).

In terms of the reaction type to photoperiod and low light intensity the barley varieties were placed into four major groups, two of which were homogeneous with respect to growth habit. These groups may thus represent the typical reactions of spring vs. winter genotypes. Spring varieties showed less variance in sensitivity to photoperiod and low light intensity compared to winter barleys, as 67% of the spring genotypes were found to have similar reaction types, while this value was only 47% for the winter barleys. Thus, in general the typical reaction of a variety with spring growth habit can be described as insensitivity to both long and short photoperiods, which may be significantly modified by low light intensity, delaying heading under a long photoperiod and hastening it under a short one. Similarly, a typical winter barley can be characterised as having relative insensitivity to a long photoperiod, where low light intensity has no effect, and sensitivity to a short photoperiod, where low light intensity has a significant delaying effect. In addition to establishing the typical reaction types of the two growth-habit groups, this study also made it possible to identify genotypes with contrasting or unusual combinations. Based on this information a more detailed experiment on a wider range of light intensities, involving barley genotypes with special reactions to light intensity, may help to dissect the role played by light intensity in determining heading, and its association with other environmental factors.

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# COMPARISON OF GERMINATION RESPONSES OF CULTIVATED WHEAT (*Triticum*) AND ITS WILD RELATIVE (*Aegilops*) SPECIES UNDER SALINITY, TEMPERATURE AND LIGHT

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The seed germination of bread (*Triticum aestivum* cv. Bezostaya 1 and Ceyhan 99) and durum (*Triticum durum* cv. Diyarbakır 81 and Fırat 93) wheat species and their wild relative species (*Aegilops biuncialis* and *Ae. triuncialis*) was compared at two light levels, nine NaCl concentrations and three alternating temperature regimes. No seeds germinated at 675 mmol/L NaCl. The highest seed germination (100%) of cultivated wheat cultivars was noted in the control at 15/25°C and 20/30°C, and that of wild wheat species in both the control and the 150 mmol/L NaCl treatment under all temperature regimes. The seed germination of bread and durum wheat cultivars was completely inhibited at or above 450 and 375 mmol/L NaCl, respectively. No *Ae. biuncialis* seeds germinated at 600 mmol/L NaCl, while seeds of *Ae. triuncialis* germinated at this concentration (38.9%) only at 20/30°C in darkness. The inhibitory effect of light on germination in all genotypes was determined in some salinity levels at 15/25°C. The optimal germination treatment for all genotypes was 15/25°C temperature regime and darkness. The inhibitory effect of high salinity on germination was greater at 25/35°C than at 15/25°C or 20/30°C. In salinity and temperature interactions, the seeds of wild wheat species were found to be more tolerant than those of wheat cultivars.

**Key words:** *Aegilops* species, germination, light, salinity, temperature, *Triticum* species/cultivars

## Introduction

Salinity and high temperature are serious environmental problems in arid and semiarid regions. The Southeast Anatolia Region of Turkey is arid, with a typical terrestrial climate. Annual rainfall is around 462 mm and mean annual evaporation 2048 mm. The mean annual temperature is 18.6°C with a minimum temperature below 0°C in February and a maximum of 46.5°C (July). The Southeast Anatolia Project is the most important irrigation project in Turkey. If efficient methods of irrigation and drainage are not applied, these agricultural

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areas are expected to be affected by salt. Agricultural areas affected by salt need amendment and the determination of the most suitable plant species able to grow in these areas.

The wild relatives (*Aegilops* L.) of wheat are found in a wide range of climatic regions and also grow on many different type of soils, in some places even on salty ones (Feldman and Sears, 1981). *Aegilops* species are suggested as potential gene resources for abiotic stress tolerance in cultivated wheat (Baalbaki et al., 2006). To protect the wild wheat gene resources, the determination of plant species and environment interactions is essential. The germination responses of plant species are often found to be dependent on a combination of environmental conditions (Carmona and Murdoch, 1995). In this sense, the comparison of seed germination profiles of closely related species under abiotic stresses is important. In this research, cultivated wheat and its wild relative species were evaluated to determine the individual and combined effects of the studied abiotic factors on seed germination and to reveal any differences in the germination profiles of *Triticum* species/cultivars and their wild relative species (*Aegilops* species).

## Materials and methods

Seeds were obtained from various agricultural research institutes (ARI) in Turkey: seeds of bread wheat (*Triticum aestivum* L.) from Anatolia ARI (Bezostaya 1) and Çukurova ARI (Ceyhan 99) and durum wheat (*Triticum durum* Desf.) from Southeast Anatolia ARI (Diyarbakır 81 and Fırat 93). Ears of the wild wheat *Aegilops biuncialis* and *Ae. triuncialis* species were collected in July 2003 from Şanlıurfa in Turkey. The mature seeds used for the experiments were one year old.

For each cultivar and species, selected seeds of uniform size, shape and colour were surface-sterilized with 3% Clorox (sodium hypochlorite) solution for 10 min. The seeds showed 100% germination in distilled water in a viability test. The seeds were placed in growth chambers under two light regimes (12 h photoperiod at 110 μmol $m^{-2}s^{-1}$, Sylvania Gro-Lux fluorescence lamp, F18W/GRO and 24 h darkness), nine NaCl concentrations [0 (distilled water), 150, 225, 300, 375, 450, 525, 600 and 675 mmol/L NaCl] and alternating temperature regimes of 15/25°C, 20/30°C and 25/35°C dark/light cycle. Six replicates of 15 seeds were used for each treatment. The seeds were germinated on two layers of filter papers soaked in solution. The germination experiments were replicated three times for each treatment, using a completely randomized block design. Germination was recorded at the end of the fifth day. A seed was considered to be germinated with the emergence of the radicle (Bewley and Black, 1994), but was not considered to be germinated if shoot growth occurred in the absence of radicle extension (Almansouri et al., 2001).

The percentage germination data were transformed to arcsine before statistical analysis (ANOVA, Duncan multiple range and Student t-test at the $P<0.05$ level of significance).

## Results

Analysis of variance indicated that there were significant ($P<0.0001$) individual effects of salinity, temperature, light, genotype and their interactions on the final percentage germination (Table 1). No seeds of the tested genotypes germinated at 675 mmol/L NaCl. Therefore, this concentration was not included in the statistical analysis.

*Table 1*
Results for analysis of variance of significant (P<0.0001) individual effects of salinity (S), temperature (T), light (L), genotype (G) and their interactions on final percentage germination

| Source | Type III sum of squares | df | Mean square | F | Significance |
|---|---|---|---|---|---|
| G | 90.815 | 5 | 18.163 | 1347.953 | 0.000 |
| S | 461582 | 7 | 65.940 | 4893.704 | 0.000 |
| T | 21.476 | 2 | 10.738 | 796.895 | 0.000 |
| L | 2.286 | 1 | 2.286 | 169.622 | 0.000 |
| G × S | 43.651 | 35 | 1.247 | 92.558 | 0.000 |
| G × T | 4.144 | 10 | 0.414 | 30.752 | 0.000 |
| S × T | 7.953 | 14 | 0.568 | 42.157 | 0.000 |
| G × S × T | 14.471 | 70 | 0.207 | 15.342 | 0.000 |
| G × T | 0.383 | 5 | 7.659E-02 | 5.684 | 0.000 |
| S × L | 1.789 | 7 | 0.256 | 18.968 | 0.000 |
| G × S × L | 3.875 | 35 | 0.111 | 8.217 | 0.000 |
| T × L | 3.315 | 2 | 1.658 | 123.012 | 0.000 |
| G × T × L | 0.257 | 10 | 2.568E-02 | 1.906 | 0.040 |
| S × T × L | 1.345 | 14 | 9.606E-02 | 7.129 | 0.000 |
| G × S × T × L | 5.356 | 70 | 7.651E-02 | 5.678 | 0.000 |

Seeds of bread wheat cultivars (Bezostaya 1 and Ceyhan 99) showed 100% germination in distilled water in the 15/25°C and 20/30°C temperature regimes (Figs. 1 and 2). In addition, the germination of Bezostaya 1 seeds was 100% even at 150 mmol/L NaCl under the 15/25°C (in darkness) and 20/30°C (in both photoperiods) temperature regimes. An increase in salinity partly or completely inhibited germination (Figs. 1 and 2). No seeds of bread wheat cultivars germinated in 450 mmol/L NaCl in any temperature regime. The germination was inhibited significantly in some salinity treatments in the 15/25°C temperature regime in light compared to darkness. In addition, there was no significant difference (P>0.05) between the light and dark treatments at 20/30°C and 25/35°C. Interestingly, germination in the light was significantly higher than in darkness in 375 mmol/L NaCl at 20/30°C and in 225 mmol/L NaCl at 25/35°C. The germination of Bezostaya 1 and Ceyhan 99 seeds at 15/25°C and 20/30°C was reduced significantly from 100% to 93.3% (Bezostaya 1) and 84.4% (Ceyhan 99) at 25/35°C in the control. Salinity-induced germination inhibition was lower at 15/25°C, and higher at 25/35°C. Germination in 300 and 375 mmol/L NaCl was significantly higher in both photoperiods at 15/25°C than at other temperatures (Figs. 1 and 2).

The highest germination (100%) in durum wheat cultivars (Diyarbakır 81 and Fırat 93) was observed in the non-saline control at 15/25°C and 20/30°C (Figs. 3 and 4). Exposure to different salinity levels resulted in a gradual decrease in percentage germination, and this reduction varied with the change in alternating temperature regime. The percentage germination progressively decreased up to 300 mmol/L NaCl. No seeds of cv. Diyarbakır 81 germinated in

375 mmol/L NaCl except in darkness at 15/25°C. Similarly, no seeds of cv. Fırat 93 germinated in 375 mmol/L NaCl. In all salinity treatments, the germination of cv. Diyarbakır 81 in darkness was significantly higher than that in light at 15/25°C. This inhibitory effect of light was observed in cv. Fırat 93 under the 15/25°C and 20/30°C alternating temperature regimes. There was no significant difference in any salinity treatment between the light and dark treatments at 25/35°C. In the light, the best seed germination temperature was 20/30°C averaged over the salinity levels. However, differences in dark-germinated seeds of cv. Diyarbakır 81 were significantly higher at salinity levels above 150 mmol/L at 15/25°C compared to other temperature regimes. In contrast, there was no significant difference in the 150 and 225 mmol/L NaCl treatment between 15/25°C and 20/30°C in dark-germinated seeds of cv. Fırat 93 (Figs. 3 and 4).

The partial germination inhibition of the wild wheat species *Ae. biuncialis* and *Ae. triuncialis* was first observed at 300 mmol/L NaCl in the light and at 375 mmol/L NaCl in darkness at 15/25°C (Figs. 5 and 6), i.e. germination was 100% up to these NaCl concentrations. However, the germination of wild wheat species was only 100% in the control and at 150 mmol/L NaCl in the 20/30°C and 25/35°C temperature regimes. Approximately 40% of *Ae. triuncialis* seeds

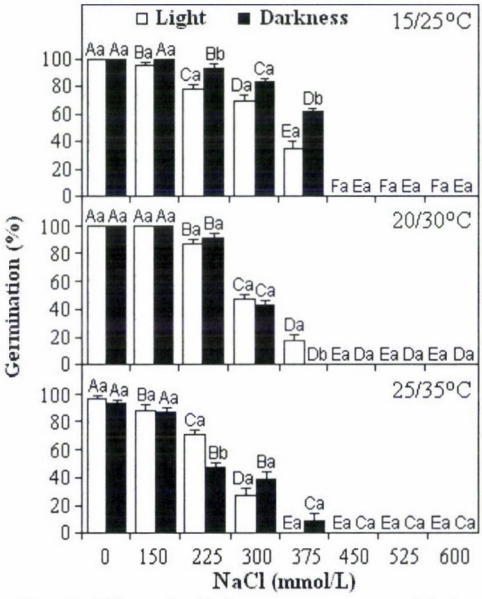



*Fig. 1.* Effect of salinity, temperature and light on the seed germination of *Triticum aestivum* L. cv. Bezostaya 1

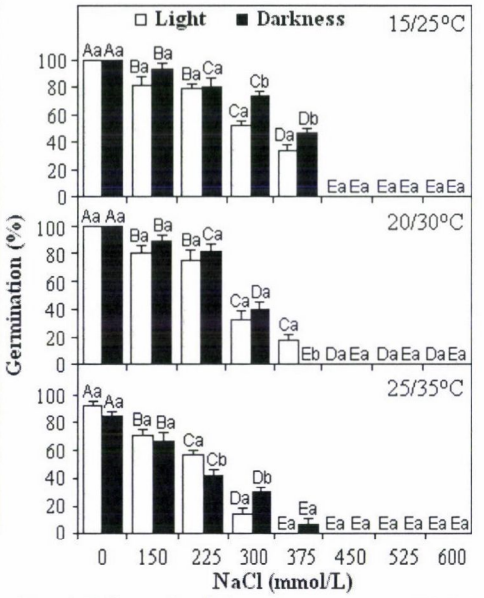



*Fig. 2.* Effect of salinity, temperature and light on the seed germination of *Triticum aestivum* L. cv. Ceyhan 99

Different capital letters at each NaCl concentration indicate significant differences ($P<0.05$) with an increase in salinity (Duncan's multiple range test), while different lower-case letters indicate significant differences ($P<0.05$) between light and darkness (Student t test). Bars represent standard errors (±SE) of means.

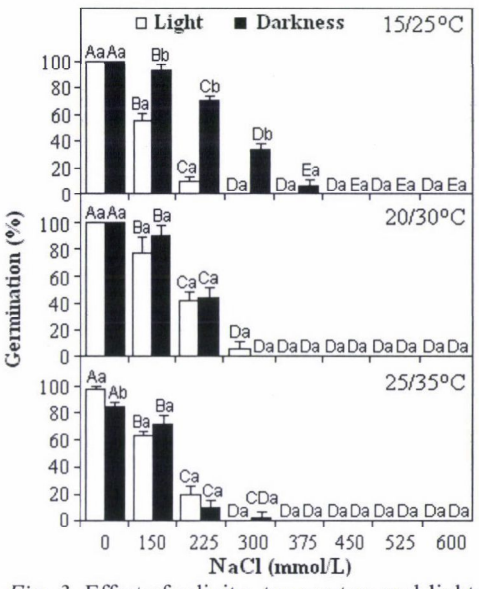



*Fig. 3.* Effect of salinity, temperature and light on the seed germination of *Triticum durum* Desf. cv. Diyarbakır 81. For meanings of letters and bars see Figure 1.

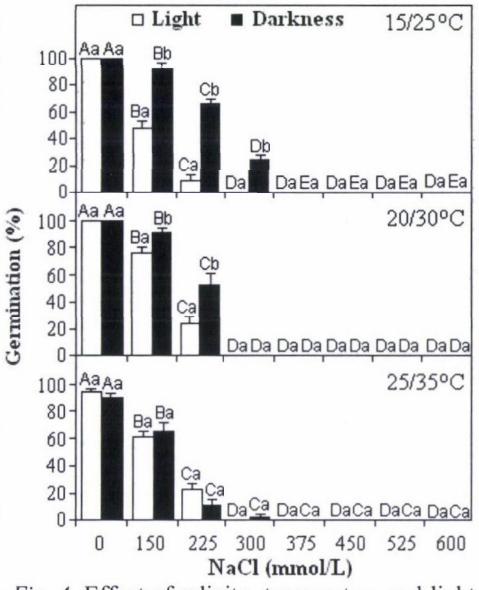



*Fig. 4.* Effect of salinity, temperature and light on the seed germination of *Triticum durum* Desf. cv. Fırat-93. For meanings of letters and bars see Figure 1.

germinated in 600 mmol/L NaCl, but only at 20/30°C in darkness, while no seeds of *Ae. biuncialis* germinated at this NaCl concentration. The inhibitory effect of light on the germination of *Ae. biuncialis* and *Ae. triuncialis* was manifested at or above 300 and 375 mmol/L, respectively, at 15/25°C. This effect was observed at 525 and 600 mmol/L NaCl in the 20/30°C temperature regime, while there was no significant difference in the 25/35°C temperature regime. Germination in light at 20/30°C was significantly higher than under other temperature regimes at 450 and 525 mmol/L NaCl. The partial inhibition of germination in the 25/35°C temperature regime was significant at or above 225 mmol/L NaCl for both photoperiods (Figs. 5 and 6).

## Discussion

Wheat is a moderately salt-tolerant crop species (Maas, 1986). Many glycophytes such as wheat were able to germinate at NaCl concentrations above 300 mmol/L (Malcolm et al., 2003), while some moderately salt-tolerant glycophytic plants (cotton and alfalfa) germinated up to 200 mmol/L NaCl (Kent and Läuchli, 1985; Rumbaugh and Pendery, 1990). Salt stress could reduce germination either by limiting water absorption by the seeds (Dodd and Donovan, 1999), by effecting the mobilization of stored reserves (Lin and Kao, 1995) or by directly affecting the structural organization or synthesis of proteins

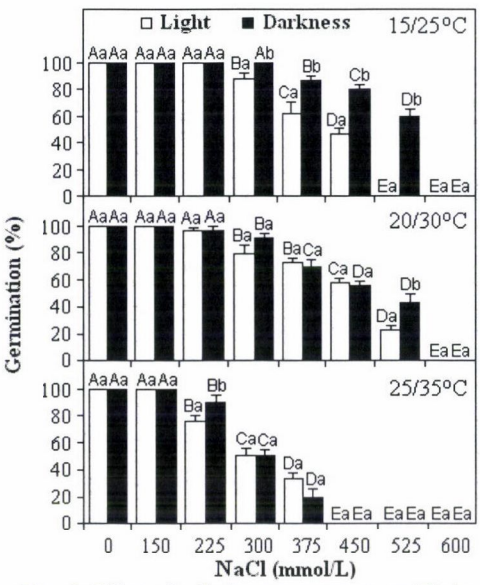



*Fig. 5.* Effect of salinity, temperature and light on the seed germination of *Aegilops biuncialis*. For meanings of letters and bars see Figure 1.

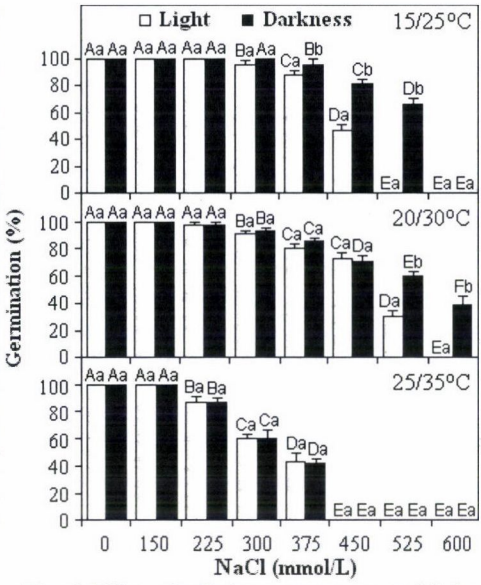



*Fig. 6.* Effect of salinity, temperature and light on the seed germination of *Aegilops triuncialis* For meanings of letters and bars see Figure 1.

in germinating embryos (Ramagopal, 1990). Salinity affects seed germination either osmotically (Welbaum et al., 1990) and/or by inducing toxicity due to the presence of excessive $Na^+$ ions (Huang and Redman, 1995). In the present research, the tested genotypes differed greatly in their response to salinity, as evaluated by the final germination percentage. However, there were similar germination profiles for two cultivars of *Triticum aestivum* (Bezostaya 1 and Ceyhan 99) and of *T. durum* (Diyarbakır 81 and Fırat 93), and for *Aegilops biuncialis* and *Ae. triuncialis* with an increase in salinity. Wild wheat species were collected from the same area. Therefore, these species might show similar germination profiles. The highest germination (100%) of cultivated wheat cultivars was obtained in the controls under the 15/25°C and 20/30°C temperature regimes. The seeds of *Aegilops* species showed 100% germination in 150 mmol/L NaCl under all temperature regimes and photoperiods. In addition, the germination of wild wheat species was 100% even in 300 mmol/L NaCl at 15/25°C in darkness. The germination of cultivated and wild wheat seeds was severely reduced by high levels of salt. Progressively stronger inhibitory effects were observed with increasing NaCl concentrations, reaching a maximum at 375 mmol/L in *Triticum durum* cultivars, 450 mmol/L in *Triticum aestivum* cultivars and 525 mmol/L in *Aegilops* species. These results indicated that the germination responses of seeds to salinity are species-specific. Ungar (1996) hypothesized that the tolerance of seeds to salinity should be considered as the ability to germinate under high salinity. In this sense, the seeds of *Aegilops* species could be regarded as highly salt-tolerant as they germinated even at 525 and 600 mmol/L NaCl.

Baskin and Baskin (1998) reported that 23 halophytic species had different germination responses in light and dark treatments. In the present study, the seeds of cultivated and wild wheat genotypes had higher percentage germination in darkness than in the light. Similarly, some researchers showed that seeds of some halophytes do not require light for germination (Andrews, 1997; Baskin and Baskin, 1998). The inhibitory effect of light on germination was markedly observed in all the tested genotypes in some salinity treatments at an alternating temperature regime of 15/25°C. The inhibition of seed germination by light was also reported for some halophytes by Thanos et al. (1994). In contrast, the germination responses of cultivated and wild wheat seeds were similar in light and darkness with an increase in the temperature. Similar results were reported by Khan and Gulzar (2003) for many halophytic perennial grasses.

The ability of seeds to germinate at increased levels of salinity was partly dependent on the test temperature (Khan and Ungar, 1999). Seeds of cultivated and wild wheat genotypes showed significant changes in germination response to salinity with the change in temperature regimes. However, the highest germination was found for an alternating temperature regime of 15/25°C in darkness at all salinities. Temperature, especially high temperature, can lead to the reduction or inhibition of germination in many seeds (Copeland and McDonald, 1995). Salinity and temperature interact in seed germination (Khan and Ungar, 1997). In the present study, the seed germination of durum cultivars (in both photoperiods) and other genotypes (in darkness) was decreased significantly at and above 300 mmol/L NaCl at 20/30°C compared to the 15/25°C temperature regime. In addition, the germination of cultivated wheat seeds at 25/35°C decreased significantly more than other temperature regimes both in the control and at all salinity levels, while this effect was observed at and above 225 mmol/L in *Aegilops* species. In this sense, the inhibitory interaction of high temperature and salinity on germination was observed at lower salt concentrations with an increase in temperature. In other words, the interaction between temperature and salinity suggests a lowering of optimum germination temperature with increasing salinity. Similar results were reported for *Atriplex semibaccata*, *Dimorphotheca sinuata*, *Senecio elegans* (De Villiers et al., 1994), *Prosopis* species (Villagra, 1997; El-Keblawy and Al-Rawai, 2005) and *Panicum turgidum* (Al-Khateeb, 2006). The inhibitory effect of salinity at high temperatures has ecological significance because it prevents seeds from germinating in salt-affected habitats and consequently avoids seedling mortality during periods when surface soil salinities are extremely high (Khan and Ungar, 1998). Contrary to these results, the seed germination of durum wheat cultivars in 150 and 225 mmol/L NaCl and of wild wheat species in 450 and 525 mmol/L NaCl at 20/30°C was significantly higher in comparison with other temperature regimes in the light treatment. Similarly, the seed germination of *Aeluropus lagopoides* at all salinities was higher at optimum temperature compared to other

temperature regimes (Gulzar and Khan, 2001). On the other hand, the germination percentage of *Ae. triuncialis* was 38.9% at the highest salt level (600 mmol/L NaCl), but only at 20/30°C in darkness. In this species, germination was completely inhibited when the temperature was further increased or decreased. A similar result was reported for the seed germination of *Aeluropus lagopoides* by Gulzar and Khan (2001).

In the present study, the ranking of the tolerance of seeds at the germination stage, in descending order, was found to be wild wheat species > bread wheat cultivars > durum wheat cultivars. The evaluation of the responses of currently cultivated wheat cultivars and their wild relatives to environmental stresses is important for improving their stress tolerance.

## Acknowledgements

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# STUDY OF THE EFFECT OF PLANT DENSITY ON THE GROWTH OF MAIZE (*Zea mays* L.) HYBRIDS USING THE RICHARDS FUNCTION

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In maize, plant density has a considerable influence on the rate of dry matter accumulation and on its partitioning between vegetative and reproductive sinks. The aim of the present research was to use the first, second and third derivatives of the Richards function (RF) for growth analysis on maize hybrids grown at various densities. In two-factorial split-plot experiments carried out in Martonvásár, Hungary in 1997–1999 the growth analysis method was used to examine the effect of six plant densities (20, 40, 60, 80, 100 and 120 thousand plants $ha^{-1}$) on the growth of three maize hybrids (Mara, Mv 355, Florencia) with different vegetation periods. Plant density had a significant effect on the dynamics of dry matter accumulation, absolute growth rate (AGR) and absolute acceleration rate (AAR). There was a significant reduction in the asymptotic maximum (A) and growth parameters (AGR, AAR) of the whole plant and of the individual plant organs (stalk, leaf, ear and grain yield), while the parameters of the leaf area index (LAI) increased significantly with a rise in the plant density. The usefulness of the RF for approximating the growth processes of maize plants and individual plant parts was confirmed statistically.

**Key words:** maize, plant density, plant growth analysis, Richards function, dry matter accumulation, leaf area index

**Abbreviations:** A, asymptotic maximum; AGR, absolute growth rate; AAR, absolute acceleration rate; D, grand period of growth; $D_L$, duration of linear growth; PGR, plant growth rate; HP, Hunt–Parsons model; LAI, leaf area index; RF, Richards function; RGR, relative growth rate.

## Introduction

Genetic improvements in maize hybrids have been associated with an increase in the plant density at which maximum grain yield is attained (i.e. the optimum plant density for grain yield) (Russell, 1974; Crosbie, 1982; Tollenaar et al., 1993; Duvick et al., 2004). Tolerance of high plant density and of other biotic and abiotic stresses, along with improved resource use efficiency,

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constitute the determinant parameters that contribute to improved productivity (Tollenaar and Lee, 2002). In general, the effect of the rate of dry matter accumulation during the silking period on kernel formation shows how plant density and environment/management factors can influence the grain yield of maize. When the plant growth rate is slow because of high density or poor environmental conditions, the growth of the ears is affected to a greater extent than that of other organs (Otegui and Andrade, 2000). At high plant densities, maize plants have a low plant growth rate (PGR) and are barren, while at low densities they have higher PGR and are prolific (Ritchie and Alagarswamy, 2003). Tollenaar et al. (1992) and Andrade et al. (1999) found that maize plants were barren when PGR averaged about 1.0 g during the 30-day period around silking. The lower proportion of barren plants in new hybrids compared to old ones appeared to be the result of higher rates of dry matter accumulation in the new hybrids. Plant density is the management practice that has the greatest impact on leaf area index (LAI) (the leaf area of a crop per unit ground area), and hence on the light interception of maize canopies (Westgate et al., 2004).

Plant growth analysis, *sensu lato*, involves the quantitative study of the performance of plants or plant components, integrated both throughout the system under study and across ecologically or agronomically meaningful intervals of time (Hunt, 1982). Growth analysis is a method for following the dynamics of net photosynthetic production as measured by the production of plant dry matter and is one approach to the analysis of yield-influencing factors and plant development. Several authors have published reviews on growth analysis techniques (Radford, 1967; Evans, 1972; Causton and Venus, 1981; Hunt, 1982). In growth analysis much attention has been recently paid to the functional approach, using suitable mathematical models to describe plant growth empirically. The calculation of growth functions allows a more complete description of plant responses to a given environmental situation (Bullock et al., 1988).

The literature on growth analysis contains tools for fitting low-order polynomials, non-linear asymptotic functions with up to four parameters, and splined curves (smoothly joined polynomials), which offer almost unlimited flexibility (Hunt and Evans, 1980; Causton and Venus, 1981; Hunt, 1982). Hunt and Parsons (1974) used a stepwise regression procedure to choose the most appropriate of the first three orders of the polynomial exponential family. The Richard function (RF), first introduced in 1959, is a highly flexible asymptotic function with a characteristic sigmoid shape. The results of Venus and Causton (1979) and Prasad and Shankar (1992) show that although there is rarely a statistical difference between the quantities derived from the polynomial exponential and Richards functions, the time trends are often more biologically meaningful when derived by fitting the RF. A full appraisal of the value of the RF in modelling plant growth was given by Causton and Venus (1981). Growth analysis using the first, second and third derivatives of the RF was presented by Nath and Moore (1992) and by Gregorczyk (1998).

Many investigations have dealt with the general growth pattern of maize. The accumulation of dry matter tends to follow a characteristic sigmoid-shaped curve (Bair, 1942; Sayre, 1948). A comparison of growth patterns in maize may be more reliable when a growth function is used for estimation (Hunt and Evans, 1980; Stamp et al., 1982; Prasad and Shankar, 1992).

The aim of the research was (i) to characterise the effect of plant density on the growth of maize hybrids with various vegetation periods and on the dynamics of their growth parameters, and (ii) to use the functional method of growth analysis to determine to what extent the first, second and third derivatives of the RF and the critical growth periods contributed to a more complex and accurate characterisation of the plant density effect and of differences between maize hybrids.

## Materials and methods

*Field procedures*

The small-plot field experiments were set up in the institute nursery in Martonvásár, Hungary (N 47°21', E 18°49') between 1997 and 1999. The soil of the experimental area is a humus-rich loam of the chernozem type with forest residues, mildly acidic in the ploughed layer, poorly supplied with available phosphorus, but with good supplies of potassium. The humus content is about 3.2%. The experiment was set up in a split-plot design (main plot: plant density, subplot: hybrid) in four replications. There were six plant densities in the main plot (1000 plants $ha^{-1}$): 20, 40, 60, 80, 100 and 120, while the subplots consisted of three maize hybrids with different vegetation periods. The maize hybrids and their FAO numbers were the following: Mara: 290, Mv 355: 390 and Florencia: 530. The seeds were sown at a row distance of 70 cm between April 18 and 28 using a Wintersteiger plot drill. The plots were overplanted and the desired densities were obtained by thinning after seedling emergence. Each plot consisted of eight 12-m rows. The plots were fertilised with 160 kg N $ha^{-1}$, 120 kg P $ha^{-1}$ and 160 kg K $ha^{-1}$. The following rainfall quantities (mm) were recorded during the vegetation period (Apr.–Sep.): 1997: 226, 1998: 502, 1999: 478. The mean temperature (°C) during the vegetation period was: 1997: 16.4, 1998: 16.3, 1999: 16.5.

*Sampling procedures*

Samples for destructive plant analysis were taken every two weeks from 17–28 days after sowing (in the four-leaf stage, V4) until physiological maturity (R6) (Ritchie et al., 1996). At each sampling date five plants from each replication of each treatment were cut at the soil surface. Samples were taken on 9 occasions in 1997, 10 in 1998 and 11 in 1999. The plants were divided into the following organs: (1) green leaf-blade, (2) stalk with leaf-sheaths, (3) tassel, (4) husks and ear shanks, (5) ear and (6) kernels. The separated plant parts were dried at 105°C in a drying cabinet for 48–96 h for the determination of dry mass. Among the morphological traits, measurements were made on plant height, stalk diameter and leaf area. The latter was recorded using a Delta-T laboratory leaf area meter.

*The Richards function*

The Richards function may be written in the form

$$W = A\,(1 \pm e^{(b - kt)})^{-1/n}$$

RF fitting provides estimates of the parameters *A, b, k* and *n*, of which *A* and *n* can be considered to be biologically meaningful. Parameter *A* gives the asymptotic maximum size of the

whole plant or plant component, while *n* describes the shape of the curve. The constant *b* has no biological significance, but is concerned purely with the positioning of the curve in relation to the time axis. Finally, *k* is a rate constant, the interpretation of which depends upon the value of *n*. Richards (1959) and Causton and Venus (1981) proposed three different combinations of parameters which have biological significance. The first of these is a weighted mean relative growth rate ($\overline{RGR}$) over the whole growth period: k / (n + 1). The second is a weighted mean absolute growth rate ($\overline{AGR}$): A k [2 (n + 2)]. The third useful parameter combination is [2 (n + 2)] / k, which represents the time required for the major portion of growth to occur and is usually described as the duration of growth (D), although this can only be approximate as it is derived from an asymptotic function.

The RF serves as a tool for growth (W) analysis by means of the absolute growth rate (AGR), i.e. dW/dt, and the relative growth rate (RGR), i.e. d(lnW)/dt. More information may be gained using the second derivative, $d^2W/dt^2$ and the third derivative, $d^3W/dt^3$, of the RF to determine the times when the second derivative time curve reaches a maximum, minimum or zero. The latter separates growth acceleration and retardation.

Nath and Moore (1992) demonstrated the use of the second and third derivatives of the RF in biological growth analysis. The second derivative gives the absolute acceleration rate (AAR) of W with respect to time. If its value is negative, it may then be called the absolute retardation rate (ARR) of W. Thus, biological growth status can be obtained from the values of $d^2W/dt^2$ at each instant of growth. Moreover, the second and the third derivatives are helpful in determining the length of the exponential and straight line phases of growth. Nath and Moore (1992) and Gregorczyk (1998) developed and implemented mathematical formulae for the delineation of both the linear and exponential phases of biological growth. A computer program for growth analysis using the first, second and third derivatives of the RF was developed by Nath and Moore (1992) as an extention of Causton's (1969) program. In the present study this computer program was used.

*Biometrical evaluation of the experimental data*

The growth analysis program devised by Nath and Moore (1992) fits the RF to the sampling data obtained from the replicated experiment. It determines the RF parameters and performs statistical tests on the goodness of fit. It calculates estimated values of W together with the confidence intervals, and provides daily values of the parameters 1/W dW/dt, $d^2W/dt^2$ and 1/W $d^2W/dt^2$. It determines the coordinates of the critical points of time ($AAR_{max}$, inflection point, $AAR_{min}$), allowing the various phases of growth to be identified. A linear function was fitted to the linear growth phase delineated by the maximum and minimum points of the AAR curve. The regression coefficient (b) of this function was used to characterise the growth rate during the linear phase ($AGR_L$). The linear function gave a good fit, with an $r^2$ value of >0.97.

The RF was fitted to values of dry matter mass for the whole plant and individual plant organs (stalk, leaf, ear, kernels) at various times over the whole growth period. Among the morphological parameters, changes in the plant height and stalk diameter as a function of plant density were also characterised using the Richards function. The dynamics of changes in the leaf area index (LAI) over time were characterised up to the maximum with the RF. The function was fitted for each hybrid, plant density and year and for each plant organ. The goodness of fit was tested using the coefficient of determination, $R^2$. The Hunt–Parsons (HP) model used to characterise the growth dynamics of the leaf area per plant fitted a third-degree polynomial exponential function to the data.

Plant density-dependent changes in the function parameters and growth parameters obtained when fitting the RF were first evaluated for each hybrid and year. In the following stage of analysis the RF parameters and the calculated growth parameters were evaluated using analysis of variance (ANOVA) in order to determine the significance of plant density and hybrid effects. ANOVA was carried out for each parameter using the split-plot model (main plot: plant density, subplot: hybrid), with the years as replications. The values of function parameters and growth parameters given in the paper are the means of the three experimental years. Trends depending on

the plant density in the value of A were determined by means of regression analysis. The plant density-dependent growth dynamics determined by RF fitting is presented on the basis of the 1999 data for maize hybrid Mv 355.

The experimental data were processed on an IBM-compatible computer using the MSTAT-C (1991) and SPSS (2001) 11.0 for Windows programs.

## Results and discussion

*Effect of plant density on the morphological characteristics of the maize plant*

The seasonal dynamics of plant height, stalk diameter and leaf area are illustrated in Figure 1 as a function of plant density. The RF was fitted to the growth data over time for plant height and stalk diameter, while the changes in leaf area were described by the HP model using a third-degree polynomial exponential function. Significant differences as a response to plant density could only be observed for these traits from the 46th day after sowing. Plant height was significantly lower at plant densities of 20 and 40 thousand plants $ha^{-1}$ than for higher plant densities. The stalk diameter decreased significantly when the plant density rose from 20 to 120 thousand. The dynamics of leaf area increase clearly revealed that the leaf area was significantly higher at a plant density of 20–80 thousand plants $ha^{-1}$ and significantly lower at 100–120 thousand plants $ha^{-1}$.

The effect of plant density on the maximum plant height and stalk diameter was characterised by the asymptotic maximum of the RF, and the maximum leaf area per plant by the maximum value of the third-degree polynomial exponential for each year and hybrid. The significance of the treatment effects was then evaluated by two-factor ANOVA. The main effects (plant density, hybrid) were significant, but the plant density × hybrid interaction was non-significant. It can be seen from the data in Table 1 that the maximum plant height changed significantly in response to plant density. Averaged over three years and over the hybrids the plant height rose from 234.2 cm to 255.9 cm as the plant density increased from 20,000 to 100,000 plants $ha^{-1}$, being slightly lower at 120,000 plants $ha^{-1}$. The opposite tendency could be observed for stalk diameter, which declined significantly from 33.03 cm at the lowest plant density to 23.58 cm at the highest.

The maximum leaf area per plant was not significantly affected by the plant density up to a density of 60,000 plants $ha^{-1}$, but at higher values it decreased significantly. Averaged over three years the maximum leaf area ranged from 6126 to 6060 $cm^2$ at plant densities of 20–60,000 plants $ha^{-1}$, after which it declined significantly to 5052 $cm^2$ in the 120,000 plants $ha^{-1}$ treatment.

Averaged over plant density there were significant differences between the maize hybrids for all three traits examined (Table 1). Hybrids with longer vegetation periods (Mv 355, Florencia) had significantly greater stalk diameter and leaf area, while this tendency was only true of Florencia in the case of plant height.

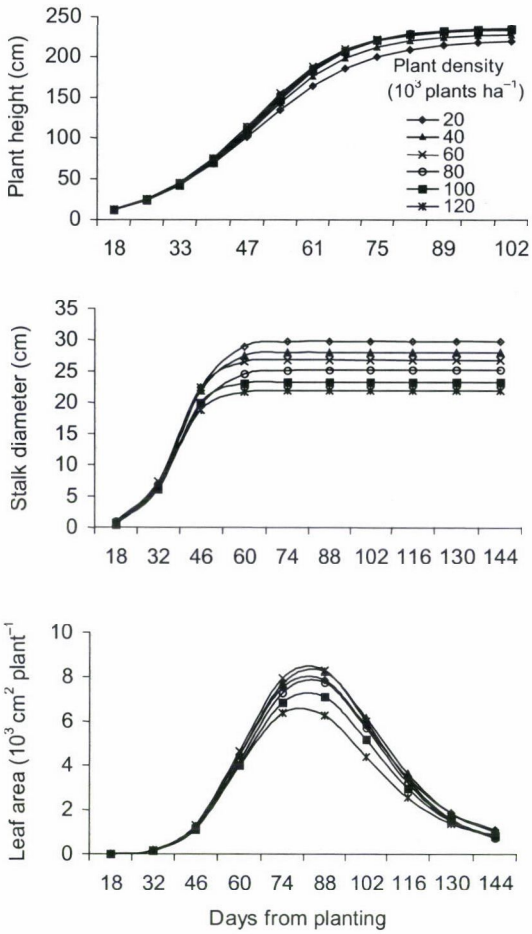



*Fig. 1.* Effect of plant density on the growth pattern of plant height, stalk diameter and leaf area using the Richards function (plant height, stalk diameter) and the HP model (leaf area)

*Table 1*

Effect of plant density ($10^3$ plants $ha^{-1}$) on the maximum values of plant height (cm), stalk diameter (cm) and leaf area ($cm^2$ $plant^{-1}$) of different maize hybrids, averaged over 1997–1999

| Plant density and hybrid | Plant height | Stalk diameter | Leaf area |
|---|---|---|---|
| 20 | 234.2 d | 33.03 a | 6126 a |
| 40 | 246.5 c | 30.05 b | 6163 a |
| 60 | 251.7 b | 28.55 c | 6060 a |
| 80 | 255.2 ab | 26.41 d | 5767 b |
| 100 | 255.9 a | 24.91 e | 5525 c |
| 120 | 251.9 b | 23.58 f | 5052 d |
| Mara | 246.2 b | 26.64 c | 5235 c |
| Mv 355 | 232.6 c | 27.66 b | 5630 b |
| Florencia | 268.9 a | 28.96 a | 6482 a |

Means followed by the same letter within a column for plant density or hybrid are not significantly different according to Duncan's multiple range test ($P \leq 0.05$)

*Dynamics of dry matter accumulation in the maize plant and in plant organs; primary and secondary parameters*

Figures 2–4 show the effect of plant density on the dynamics of dry matter accumulation, absolute growth rate and absolute acceleration rate for the whole plant, the leaves and the grain yield of maize hybrid Mv 355, determined by fitting the RF. The various parts of these figures exhibit the effect of plant density on the dynamics of dry matter accumulation. The sigmoid growth dynamics consists of an initial exponential phase, followed by a linear phase of longer duration. The linear phase is followed by a decline, until a steady state (physiological maturity) is reached. It is clear that an increase in the plant density had an influence on the dynamics of dry matter accumulation, causing a substantial decrease in the whole plant and in the individual organs. The difference in response to plant density was also perceptible in the magnitude of the final dry matter production. Plant density had no significant effect on total dry matter accumulation at the 1st and 2nd sampling dates (18 and 31 days after sowing). From the fourth sampling onwards there was a significant difference in dry matter production at all plant densities. The inflection point ($P_i$) of the curve, which simultaneously corresponded to maximum growth rate and zero acceleration, occurred at about the 74th day of growth (in the middle of silking). The linear phase of growth ended at about the 93rd–101st day of vegetation (point $P_2$).

The effect of plant density on the dynamics of LAI, absolute growth rate and absolute acceleration rate is illustrated in Figure 5, which shows that the LAI increased proportionately with the increase in the plant density from the 32nd day after sowing.

Among the primary parameters of the RF, changes in the asymptotic maximum (A) gave a good characterisation of the effect of plant density and hybrid on dry matter production and LAI. The results of two-factor ANOVA showed that the main effects (plant density, hybrid) and the plant density × hybrid interaction were all significant for the whole plant, for the individual plant organs and for LAI. The values of the mean squares, however, revealed that the interaction was far less important than the main effects. The effect of plant density on the asymptotic maximum values (A) for the whole plant, the individual plant organs and the leaf area index is illustrated in Table 2.

The asymptotic maximum (A) of the total dry matter per plant was significantly reduced when the plant density increased. Averaged over the hybrids the following values (g plant$^{-1}$) were found at various plant densities ($10^3$ plants ha$^{-1}$): 20: 544.2, 40: 431.0, 60: 345.0, 80: 295.2, 100: 234.9, 120: 196.5. As regards the differences between the hybrids, the dry matter production per plant increased significantly with the length of the vegetation period. Averaged over the plant densities the following values were recorded (g plant$^{-1}$): Mara: 297.2, Mv 355: 324.9, Florencia: 403.6. The significant plant density × hybrid interaction can be attributed to the fact that the greater mass per plant in the hybrid Florencia declined at a greater rate than that of the other two hybrids up to a plant density of 80,000 plants ha$^{-1}$, after which the slope of the curve was similar for all three hybrids (Fig. 6).

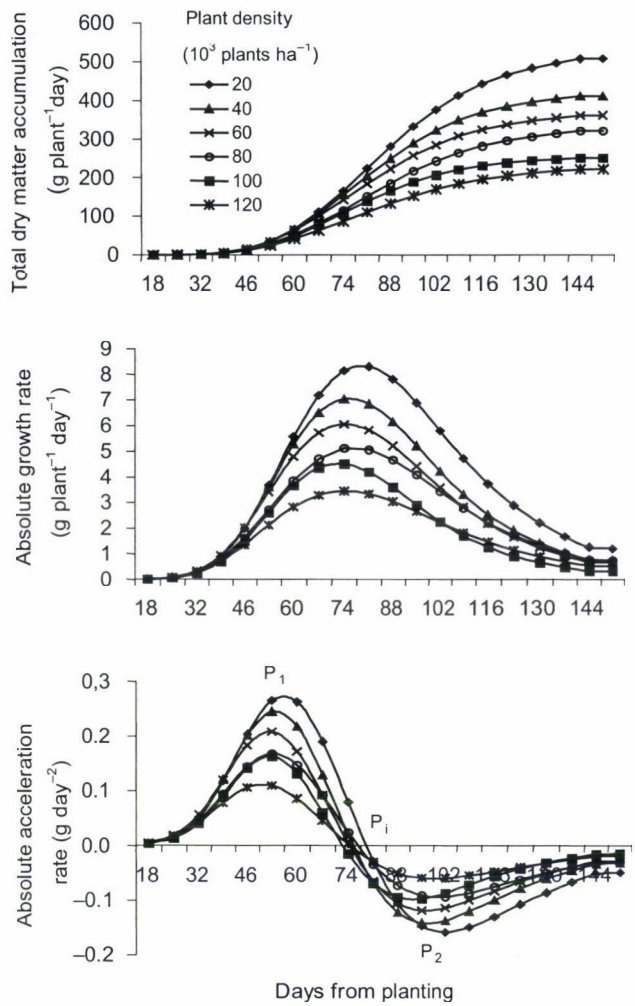



*Fig. 2.* Effect of the plant density on the dynamics of total dry matter accumulation, absolute growth rate and absolute acceleration rate of the maize hybrid Mv 355 in 1999, as determined by fitting the Richards function. $P_1$, $P_i$ show the critical points ($AAR_{max}$, inflection point, $AAR_{min}$) of growth

There was a significant reduction in the asymptotic maximum (A) of grain yield as the plant density increased, but the magnitude of the reduction varied with the hybrid. The following values (g plant$^{-1}$) were recorded at each plant density ($10^3$ plants ha$^{-1}$), averaged over the hybrids: 20: 280.0, 40: 215.3, 60: 169.4, 80: 147.8, 100: 116.7, 120: 103.6. There was no significant difference between the grain yield maximums recorded at plant densities of 100 and 120 thousand plants ha$^{-1}$. As regards the differences between the hybrids, no significant difference was observed between the grain yields of hybrids Mara and Mv 355, while the maximum (A) grain yield of Florencia was significantly the highest (g plant$^{-1}$): Mara: 152.5, Mv 355: 162.8, Florencia: 201.1.

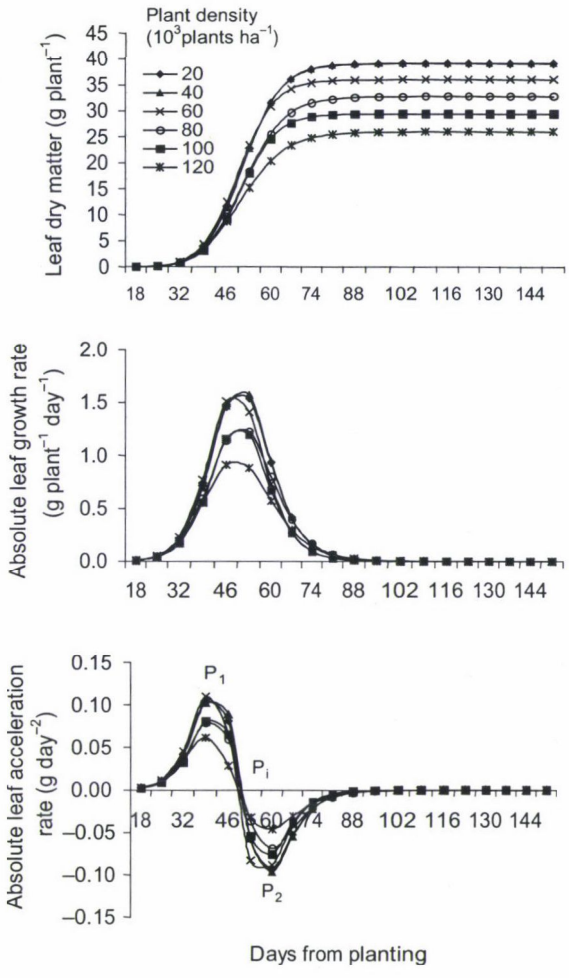



*Fig. 3.* Effect of the plant density on the dynamics of dry matter accumulation, absolute growth rate and absolute acceleration rate in the leaves of the maize hybrid Mv 355 in 1999, as determined by fitting the Richards function. $P_1$, $P_i$ and $P_2$ show the critical points ($AAR_{max}$, inflection point, $AAR_{min}$) of growth

The leaf mass also decreased significantly as the plant density increased, but to a lesser extent than observed for the other plant organs. Averaged over the hybrids the leaf mass (g plant$^{-1}$) at each plant density ($10^3$ plants ha$^{-1}$) was as follows: 20: 41.78, 40: 39.71, 60: 36.37, 80: 32.71, 100: 29.49, 120: 26.61. Hybrids with longer vegetation periods were found to have greater leaf mass per plant (g plant$^{-1}$): Mara: 30.62, Mv 355: 33.64, Florencia: 39.07. The plant density × hybrid interaction for leaf mass could be described by a linear function, the steepness (b regression coefficient) of which was as follows for the individual hybrids: Mara: –0.120, Mv 355: –0.157, Florencia: –0.195. As the plant density increased, the reduction in the asymptotic maximum for the stalks and ear yield was similar to that observed for the whole plant and for the grain yield (Table 2).

*Table 2*
Effect of plant density ($10^3$ plants $ha^{-1}$) on the asymptotic maximum values (A) for the dry matter production of maize plants, plant organs and leaf area index (LAI) when fitting the Richards function (average of 3 years)

| Maize hybrid | Plant density | | | | | |
|---|---|---|---|---|---|---|
| | 20 | 40 | 60 | 80 | 100 | 120 |
| | | | Whole plant, g $plant^{-1}$ | | | |
| Mara | 440.2 | 382.2 | 308.2 | 258.9 | 212.5 | 181.0 |
| Mv 355 | 501.8 | 392.8 | 331.8 | 295.1 | 235.5 | 192.2 |
| Florencia | 690.6 | 517.9 | 395.0 | 331.7 | 270.2 | 216.3 |
| LSD (0.05)† = 54.9 | | | | | | |
| | | | Stalk mass, g $plant^{-1}$ | | | |
| Mara | 102.2 | 90.7 | 79.3 | 66.3 | 57.6 | 51.7 |
| Mv 355 | 106.5 | 97.0 | 87.2 | 71.7 | 63.2 | 53.0 |
| Florencia | 127.9 | 112.4 | 100.1 | 83.1 | 73.5 | 61.7 |
| LSD (0.05)† = 8.1 | | | | | | |
| | | | Leaf mass, g $plant^{-1}$ | | | |
| Mara | 36.7 | 35.1 | 31.2 | 29.1 | 25.7 | 25.9 |
| Mv 355 | 40.4 | 38.5 | 36.3 | 32.6 | 29.5 | 24.6 |
| Florencia | 48.3 | 45.5 | 41.6 | 36.5 | 33.3 | 29.3 |
| LSD (0.05)† = 2.73 | | | | | | |
| | | | Ear mass, g $plant^{-1}$ | | | |
| Mara | 267.0 | 221.0 | 184.0 | 157.1 | 128.1 | 106.0 |
| Mv 355 | 292.9 | 239.1 | 196.5 | 171.8 | 138.4 | 109.9 |
| Florencia | 422.5 | 302.7 | 231.7 | 186.5 | 149.0 | 119.7 |
| LSD (0.05)† = 29.4 | | | | | | |
| | | | Grain yield, g $plant^{-1}$ | | | |
| Mara | 224.7 | 189.1 | 153.6 | 134.5 | 108.4 | 105.0 |
| Mv 355 | 251.5 | 201.2 | 162.8 | 147.9 | 114.5 | 98.8 |
| Florencia | 363.8 | 255.7 | 191.8 | 161.1 | 127.1 | 107.2 |
| LSD (0.05)† = 26.0 | | | | | | |
| | | | LAI | | | |
| Mara | 1.08 | 2.14 | 3.14 | 4.02 | 5.03 | 5.51 |
| Mv 355 | 1.13 | 2.31 | 3.42 | 4.44 | 5.23 | 5.78 |
| Florencia | 1.37 | 2.76 | 4.05 | 5.13 | 6.01 | 6.68 |
| LSD (0.05)† = 0.44 | | | | | | |

† LSD values for the comparison of two plant density means within rows or columns for each asymptotic maximum.

The asymptotic maximum of leaf area index ($LAI_{max}$) exhibited a tendency opposite to that for dry matter production, i.e. its value increased significantly with an increase in plant density. Averaged over the hybrids the following values were recorded for the various plant densities ($10^3$ plants $ha^{-1}$): 20: 1.196, 40: 2.405, 60: 3.536, 80: 4.530, 100: 5.422, 120: 5.993. For the individual hybrids the $LAI_{max}$ value increased parallel with the length of the vegetation period: Mara: 3.49, Mv 355: 3.72, Florencia: 4.33. The plant density × hybrid interaction could be expressed by a quadratic function (Fig. 6).

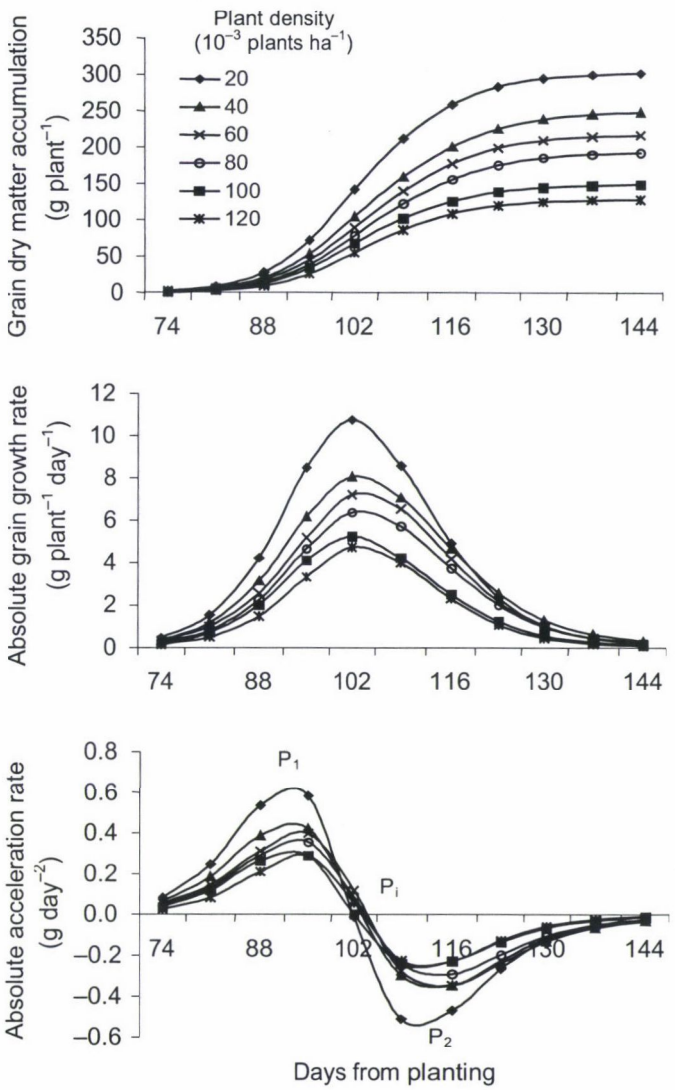



*Fig. 4.* Effect of the plant density on the dynamics of dry matter accumulation, absolute growth rate and absolute acceleration rate in the grain yield of the maize hybrid Mv 355 in 1999, as determined by fitting the Richards function. $P_1$, $P_i$ and $P_2$ show the critical points ($AAR_{max}$, inflection point, $AAR_{min}$) of growth

On the basis of two-factor ANOVA, plant density and the hybrid were found to have a significant effect on the AGR and AAR parameters, but the plant density × hybrid interaction was not significant. Changes in these parameters are thus discussed separately as a function of plant density and hybrid.

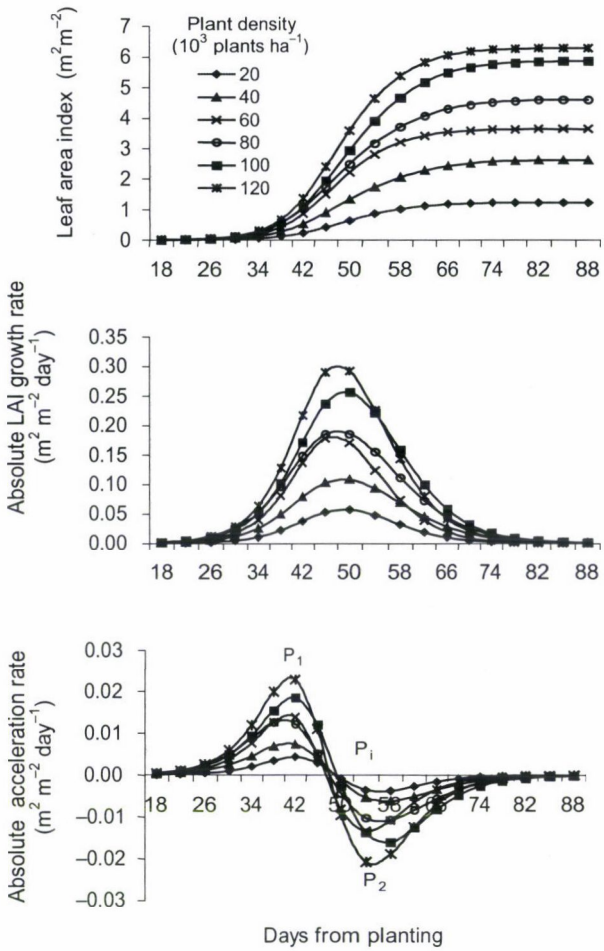



*Fig. 5.* Effect of the plant density on the dynamics of LAI, its absolute growth rate and absolute acceleration rate for the maize hybrid Mv 355 in 1999, as determined by fitting the Richards function. $P_1$, $P_i$ and $P_2$ show the critical points ($AAR_{max}$, inflection point, $AAR_{min}$) of growth

Among the secondary parameters the plant density had a significant effect on the mean absolute growth rate ($\overline{AGR}$) (Table 3). The $\overline{AGR}$ values of dry matter production for the individual plant organs and for the whole plant declined significantly as the plant density increased, but the significance of the difference between consecutive plant density levels differed for the various plant parts. The $\overline{AGR}$ value for the whole plant decreased from 5.77 to 2.17 as the plant density rose from 20 to 120 thousand plants $ha^{-1}$. The highest value of $\overline{AGR}$ was recorded for the grain yield, which decreased from 6.97 g $day^{-1}$ to 1.88 g $day^{-1}$ as the plant density increased. Similar $\overline{AGR}$ values were recorded for the ear yield, while the stalk $\overline{AGR}$ value declined from 2.76 to 1.38 g $day^{-1}$ and that of the leaf mass from 1.01 to 0.71 g $day^{-1}$ with an increase in plant density. The $\overline{AGR}$ value of LAI rose from 0.0292 to 0.1574 $cm^2$ $day^{-1}$ with

increasing plant density. Significant differences between the $\overline{AGR}$ values of the hybrids were only observed for the whole plant, the ear and the grain yield (Table 4). There were no differences between the $\overline{AGR}$ values of Mara and Mv 355, while that of Florencia was significantly higher. Little difference was observed between the hybrids for the $\overline{AGR}$ value of LAI.

*Table 3*

Effect of plant density ($10^3$ plants $ha^{-1}$) on the absolute growth rate (AGR) and absolute acceleration rate (AAR) of the whole plant and plant organs of maize hybrids, calculated by fitting the Richards function (average of 3 years)

| Maize plant and plant organs | Plant density | | | | | |
|---|---|---|---|---|---|---|
| | 20 | 40 | 60 | 80 | 100 | 120 |
| | | | $AGR_{max}$, g $day^{-1}$ | | | |
| Leaves | 1.62 a | 1.53 a | 1.48 a | 1.30 b | 1.17 b | 1.13 b |
| Stalk | 4.29 a | 3.75 ab | 3.52 b | 2.81 c | 2.53 cd | 2.19 d |
| Ear | 10.20 a | 8.43 b | 6.88 c | 5.83 cd | 4.66 de | 3.97 e |
| Grain yield | 11.69 a | 9.32 ab | 7.79 bc | 5.98 cd | 4.72 d | 3.81 d |
| Maize plant | 8.62 a | 6.95 b | 5.83 bc | 4.58 cd | 3.90 d | 3.25 d |
| LAI* | 0.0472 e | 0.0925 d | 0.1417 c | 0.1741 c | 0.2091 b | 0.2463 a |
| | | | $AGR_L$, g $day^{-1}$ | | | |
| Leaves | 1.46 a | 1.41 a | 1.35 a | 1.19 b | 1.07 b | 1.03 b |
| Stalk | 3.92 a | 3.44 ab | 3.24 b | 2.57 c | 2.31 cd | 2.00 d |
| Ear | 9.51 a | 7.69 b | 6.27 bc | 5.35 cd | 4.26 de | 3.63 e |
| Grain yield | 10.73 a | 8.59 ab | 7.10 bc | 5.45 cd | 4.33 d | 3.47 d |
| Maize plant | 7.88 a | 6.37 b | 5.21 bc | 4.18 cd | 3.56 d | 2.96 d |
| LAI* | 0.0449 e | 0.08662 d | 0.1299 c | 0.1596 bc | 0.1912 b | 0.2258 a |
| | | | AGR, g $day^{-1}$ | | | |
| Leaves | 1.01 a | 0.95 a | 0.94 a | 0.81 b | 0.76 b | 0.71 b |
| Stalk | 2.76 a | 2.44 ab | 2.24 b | 1.78 c | 1.63 cd | 1.38 d |
| Ear | 6.63 a | 5.35 b | 4.45 bc | 3.67 cd | 2.97 de | 2.48 e |
| Grain yield | 6.97 a | 5.81 ab | 4.85 bc | 3.77 cd | 3.05 de | 1.88 e |
| Maize plant | 5.77 a | 4.66 b | 3.83 bc | 3.07 cd | 2.51 d | 2.17 d |
| LAI* | 0.0292 e | 0.0580 d | 0.0899 c | 0.1102 c | 0.1344 b | 0.1574 a |
| | | | $AAR_{max}$, g $day^{-2}$ | | | |
| Leaves | 0.101 a | 0.095 a | 0.098 a | 0.106 a | 0.075 a | 0.077 a |
| Stalk | 0.257 a | 0.225 a | 0.215 ab | 0.169 bc | 0.153 c | 0.133 c |
| Ear | 0.579 a | 0.502 ab | 0.413 bc | 0.353 cd | 0.284 d | 0.258 d |
| Grain yield | 0.813 a | 0.661 ab | 0.563 abc | 0.407 bcd | 0.326 cd | 0.273 d |
| Maize plant | 0.272 a | 0.223 b | 0.189 bc | 0.147 cd | 0.132 d | 0.110 d |
| LAI* | 0.0030 d | 0.0057 cd | 0.0092 bc | 0.0110 bc | 0.0134 ab | 0.0169 a |
| | | | $AAR_{min}$, g $day^{-2}$ | | | |
| Leaves | –0.114 a | –0.132 a | –0.118 a | –0.108 a | –0.078 a | –0.092 a |
| Stalk | –0.297 a | –0.246 abc | –0.263 ab | –0.205 bc | –0.184 bc | –0.171 c |
| Ear | –0.461 a | –0.496 a | –0.392 ab | –0.356 ab | –0.261 b | –0.258 b |
| Grain yield | –0.841 a | –0.743 ab | –0.593 abc | –0.374 abc | –0.296 bc | –0.208 c |
| Maize plant | –0.194 a | –0.158 ab | –0.126 abc | –0.097 bc | –0.085 c | –0.073 c |
| LAI* | –0.0038 d | –0.0056 cd | –0.0121 bc | –0.0152 ab | –0.0147 ab | –0.0202 a |

Means within rows of the same plant or plant organ and growth indices followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$); *Dimensions for LAI are $cm^2cm^{-2}$ $day^{-1}$ for AGR and $cm^2cm^{-2}day^{-2}$ for AAR

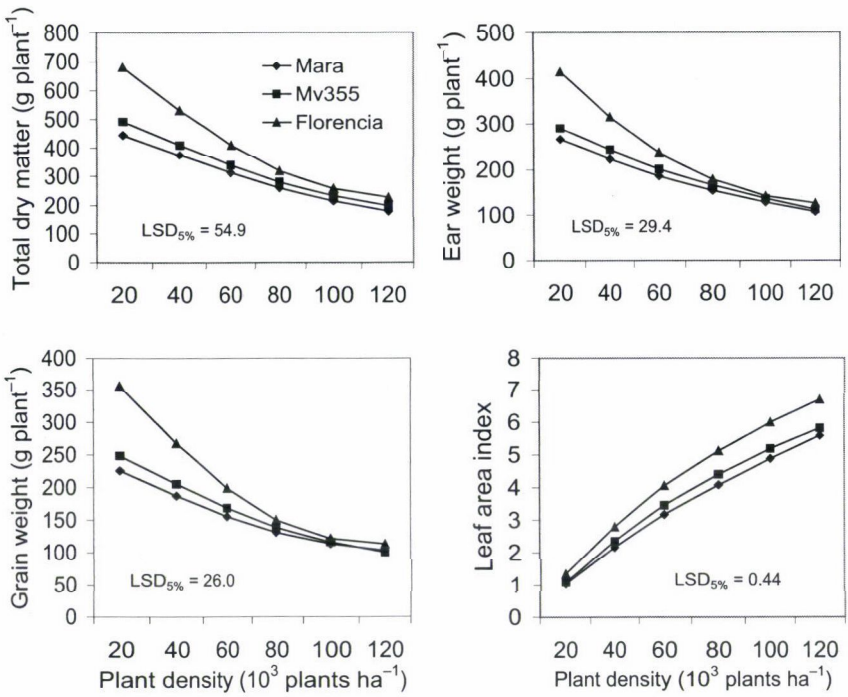



*Fig. 6.* Trends in the asymptotic maximum as affected by plant density and hybrid, when fitting a quadratic function to the A values of the Richards function (R values were >0.995)

Among the secondary parameters, the plant density had no significant effect on the mean relative growth rate ($\overline{RGR}$) for the dry matter production of either the whole plant or the individual plant organs (data not shown). The $\overline{RGR}$ values of the hybrids differed significantly, however, with the exception of the grain yield (Table 4). Mara, the hybrid with the shortest vegetation period, had the highest $\overline{RGR}$ value, while that of hybrids with longer vegetation periods was significantly lower. The $\overline{RGR}$ values of Mv 355 and Florencia only differed significantly for the leaf mass.

Plant density had no significant effect on the third of the secondary parameters tested (D, duration of growth) for either the whole plant or the individual plant organs (data not shown), but significant differences were observed between the hybrids (Table 5). In general the duration of growth increased with the length of the vegetation period. The duration of growth for the whole plant ranged from 81.7 to 100.8 days, depending on the hybrid, while the growth of the ear yield had a duration of 40.4–47.8 days. There was no significant difference in the duration of growth for the grain yield. The duration of growth increased with the length of the vegetation period for the stalk and leaves. A similar tendency was observed for the linear phase of growth ($D_L$) as for the main growth period.

*Table 4*
Absolute growth rate (AGR; g day$^{-1}$), relative growth rate (RGR; mg g$^{-1}$ day$^{-1}$) and absolute acceleration rate (AAR; g day$^{-1}$) of the whole plant and plant organs of different maize hybrids, calculated by fitting the Richards function (average of 3 years)

| Hybrid and maize organs | Growth parameters | | | | | |
|---|---|---|---|---|---|---|
| | $AGR_{max}$ | $\overline{AGR}$ | $AGR_L$ | $\overline{RGR}$ | $AAR_{max}$ | $AAR_{min}$ |
| Maize plant | | | | | | |
| Mara | 5.43 b | 3.59 b | 4.91 b | 43.1 a | 0.185 a | –0.131 a |
| Mv355 | 5.14 b | 3.44 b | 4.69 b | 38.9 b | 0.165 a | –0.112 a |
| Florencia | 6.00 a | 4.02 a | 5.48 a | 37.6 b | 0.186 a | –0.124 a |
| Leaves | | | | | | |
| Mara | 1.48 a | 0.88 a | 1.35 a | 84.6 a | 0.103.a | –0.061 a |
| Mv355 | 1.30 a | 0.84 a | 1.20 a | 78.2 b | 0.081 a | –0.088 b |
| Florencia | 1.33 a | 0.87 a | 1.21 a | 72.0 c | 0.091 a | –0.072 b |
| Stalk | | | | | | |
| Mara | 3.25 a | 2.08 a | 2.97 a | 82.3 a | 0.213 a | –0.267 a |
| Mv355 | 3.06 a | 1.93 a | 2.80 a | 73.6 b | 0.181 b | –0.223 ab |
| Florencia | 3.24 a | 2.10 a | 2.96 a | 70.4 b | 0.182 b | –0.193 b |
| Ear | | | | | | |
| Mara | 6.59 ab | 4.09 b | 6.02 ab | 76.4 a | 0.415 a | –0.467 a |
| Mv355 | 6.13 b | 4.00 b | 5.60 b | 73.0 ab | 0.363 a | –0.288 b |
| Florencia | 7.26 a | 4.69 a | 6.74 a | 70.7 b | 0.417 a | –0.357 ab |
| Grain yield | | | | | | |
| Mara | 6.73 b | 4.09 b | 6.13 b | 83.9 a | 0.487 ab | –0.521 a |
| Mv355 | 6.65 b | 4.14 b | 6.07 b | 81.7 a | 0.464 b | –0.428 a |
| Florencia | 8.27 a | 4.93 a | 7.64 a | 82.3 a | 0.570 a | –0.577 a |
| Leaf area index* | | | | | | |
| Mara | 0.1648 a | 0.1033 a | 0.1526 a | 0.0843 a | 0.0115 a | –0.0165 a |
| Mv355 | 0.1435 b | 0.0897 b | 0.1320 b | 0.0763 b | 0.0089 b | –0.0116 ab |
| Florencia | 0.1471 b | 0.0966 ab | 0.1344 b | 0.0738 b | 0.0091 b | –0.0077 b |

Means within columns for the same plant or plant organ followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$); * Units are cm$^2$ cm$^{-2}$ d$^{-1}$ for AGR, day$^{-1}$ for RGR and cm$^2$ cm$^{-2}$ day$^{-2}$ for AAR.

*Curves and parameters derived from fitted Richards functions*

The dynamics of absolute growth rate (AGR) for maize plants and plant organs was characterised by a bell-shaped curve, the course and particularly the maximum value of which differed significantly at each plant density (Figs 2–4). The acceleration/retardation curves in Figures 2–4 show just how dynamic the growth of the maize plant and of different plant parts actually is. It can be seen from these graphs that AAR becomes negative, i.e. retardation begins, at the instant when AGR begins declining. During plant growth, there are three characteristic critical moments that can be marked as $P_1$, $P_i$ and $P_2$ on the graphs. The point $P_1$ marks the maximum acceleration of growth and the first inflection of the growth rate curve. At the point $P_i$ the growth rate attains its maximum value. At the point $P_2$, maximum deceleration takes place, i.e. maximum negative acceleration of growth, while at the same time there is a second inflection of the growth rate curve. Points $P_1$ and $P_2$ separate the entire growth period into three phases: the exponential phase, the linear phase (from $P_1$ to $P_2$) and the aging phase.

*Table 5*

Times at which the maximum and minimum values of the absolute acceleration rate ($AAR_{max}$, $AAR_{min}$) and the inflection point ($P_i$) occur, as well as the grand period of growth (D) and the duration of linear growth ($D_L$) for different maize hybrids, calculated by fitting the Richards function (average of 3 years)

| Hybrid and maize organs | Growth parameters | | | | |
|---|---|---|---|---|---|
| | Time of $AAR_{max}$ | Time of inflection point | Time of $AAR_{min}$ | D | $D_L$ |
| Whole plant | | days from sowing | | days | |
| Mara | 54.4 a | 73.5 b | 92.6 b | 81.7 b | 38.2 b |
| Mv355 | 52.4 b | 74.9 b | 97.4 a | 95.7 a | 45.0 a |
| Florencia | 53.6 ab | 77.3 a | 101.0 a | 100.8 a | 47.4 a |
| Leaves | | | | | |
| Mara | 45.7 a | 51.9 ab | 58.1 b | 32.8 c | 12.4 c |
| Mv355 | 42.8 b | 50.9 b | 59.1 b | 39.6 b | 16.3 b |
| Florencia | 43.2 b | 53.0 a | 62.8 a | 47.1 a | 19.6 a |
| Stalk | | | | | |
| Mara | 52.4 a | 59.9 c | 67.4 c | 35.7 b | 15.0 c |
| Mv355 | 53.0 a | 61.6 b | 70.2 b | 40.4 a | 17.2 b |
| Florencia | 53.7 a | 63.3 a | 72.9 a | 44.0 a | 19.3 a |
| Ear | | | | | |
| Mara | 88.5 b | 97.9 c | 107.2 c | 40.4 b | 18.8 a |
| Mv355 | 89.2 b | 99.9 b | 110.6 b | 45.7 a | 21.4 a |
| Florencia | 92.2 a | 103.3 a | 114.4 a | 47.8 a | 21.8 a |
| Grain yield | | | | | |
| Mara | 92.4 c | 100.4 c | 108.5 c | 35.9 a | 16.1 a |
| Mv355 | 94.4 b | 102.7 b | 111.1 b | 37.0 a | 16.8 a |
| Florencia | 98.0 a | 106.3 a | 114.6 a | 36.8 a | 16.4 a |

Means within columns for the same plant or plant organ and growth indices followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$)

Table 3 demonstrates the absolute growth rates (AGR) and absolute acceleration rates (AAR) as affected by plant density. There was a significant decrease in the maximum value of the absolute growth rate ($AGR_{max}$) for both the whole plant and the individual plant parts as the plant density increased. The value of $AGR_{max}$ for the whole plant declined from 8.62 to 3.25 g plant$^{-1}$ day$^{-1}$ when the plant density rose from 20 to 120 thousand plants ha$^{-1}$. The highest value of $AGR_{max}$ was recorded for grain yield and ear yield and the lowest for leaf mass. The $AGR_{max}$ value for grain yield dropped from 11.69 to 3.81 g plant$^{-1}$ day$^{-1}$ when the plant density rose from 20 to 120 thousand plants ha$^{-1}$. Great similarity was exhibited by the $AGR_{max}$ values for ear and grain yield. The $AGR_{max}$ value for leaf mass did not change significantly between plant density values of 20 to 60 thousand plants ha$^{-1}$ (decreasing from 1.62 to 1.48), but decreased significantly from 80,000 plants ha$^{-1}$. The $AGR_{max}$ value for maize stalks decreased from 4.29 to 2.19 g day$^{-1}$ as the plant density rose. Research showed that during the straight line phase of growth (between the times when $d^2W/dt^2$ was maximum and minimum) the value of AGR ($AGR_L$) was very close

to that of $AGR_{max}$, indicating that during this period the growth of the maize plant approaches the maximum value of the absolute growth rate (Table 3).

The absolute acceleration rate ($AAR_{max}$) was also a good indicator of the effect of plant density. Its value for grain yield was 0.813 g $day^{-2}$ at the lowest plant density, decreasing to 0.273 g $day^{-2}$ at the highest plant density. The same values for the whole plant were 0.272 and 0.110 g $day^{-2}$, respectively. A similar tendency was seen as the result of plant density in the $AAR_{min}$ values. An analysis of the differences in the AGR and AAR parameters for each hybrid revealed the same significant differences between the hybrids as described for $\overline{AGR}$. No difference was observed between the hybrids in the $AAR_{max}$ values for the mass of the whole plant, the leaf and the ear. For the mass of the grain yield, the highest $AAR_{max}$ value (0.570 g $day^{-2}$) was found for Florencia, the hybrid with the longest vegetation period (Table 4).

The plant density had a significant effect on the occurrence of the critical times ($AAR_{max}$, inflection point, $AAR_{min}$) for the maize plant, but the density effect was not significant in the case of ear dry matter, leaf dry matter and grain yield (data not shown). The $AAR_{max}$ of the whole maize plant was recorded at 57.9 days from sowing at the lowest density, while this point came significantly earlier, at 49.8 days, at the highest plant density. For the grain yield the time of $AAR_{max}$ varied from 95.4 to 93.2 days, but the density effect was not significant. The inflection point of the whole maize plant (total dry matter) was observed at 80.3 days at the lowest plant density, being significantly earlier, at 70.8 days, at the highest plant density. For the grain yield the inflection point was observed at almost the same time (between 102.3 and 103.8 days). $AAR_{min}$ was recorded significantly later, at 102.8 days, at 20,000 plants $ha^{-1}$ than at 120,000 plants $ha^{-1}$ (91.8 days) for the whole maize plant, while it was not significantly different for grain yield (ranging from 110.3 to 111.8 days).

Table 5 lists the occurrence of the critical moments ($AAR_{max}$, inflection point, $AAR_{min}$) for the whole plant and the individual plant organs in various hybrids, together with the grand period of growth (D) and the duration of the linear growth phase ($D_L$). It can be seen that the critical moments occurred later in the hybrid Florencia, which had the longest vegetation period.

## Conclusions

The usefulness of the Richards function for the approximation of the growth process in the whole maize plant and in individual plant parts was confirmed statistically. In general, the fitting of the Richards function caused no problems, but when the data did not adequately represent all the growth stages of the plant or plant organs, fitting was not possible. The general magnitudes of the standard errors of the parameters reflected the size of the variability of the underlying data.

The analysis of plant density effects on the growth of the whole maize plant and of individual plant organs as presented in this paper demonstrates the use of derivatives of the Richards function initially fitted to plant growth data. Uniquely, the second derivative gives specific answers to questions concerning the growth status at any instant after emergence. Conclusions can be made about the history of crop growth on a day-by-day basis by simply studying these curves and evaluating them for critical points in time.

The effect of the plant density on the growth of the whole plant and the individual plant organs could be accurately characterised using the seasonal dynamics and parameters of the absolute growth rate (AGR) and the absolute growth acceleration (AAR). An increase in plant density reduced the growth rate, particularly that of the ear yield and grain yield. These results confirm those reported by Tollenaar et al. (1992), Andrade et al. (1999) and Ritchie and Alagarswamy (2003). It was found that growth during the linear phase ($AGR_L$) took place at close to the maximum rate ($AGR_{max}$). The maintenance of linear growth is extremely important in crop production, as the duration and rate of this phase of growth is decisive for the yield (Stamp and Kullman, 1984; Gardner et al., 1985). The plant growth rate (AGR) and acceleration rate (AAR) during critical periods could be useful variables for studying the mechanism of kernel set and ear plasticity and for defining threshold values for ear abortion, prolificacy and limitations on ear morphogenesis.

Growth analysis provides an excellent opportunity to monitor the independent and interactive effects of various factors affecting maize yields, and opens the way to managing these factors in integrated systems. The diagnosis of growth-limiting factors and the forecasting of grain yield through growth analysis should significantly improve site-specific farming (Machado et al., 2002). Although the growth analysis parameters presented here refer to individual plants, they can easily be converted to plant stands, making the method suitable for the determination of optimum plant density and maximum yield.

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# SULPHUR MANAGEMENT IN MOONG (*Phaseolus aureus* L.) AND RAYA (*Brassica juncea* L.) CROPS

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Studies on the sulphur requirements of crops have largely been restricted to single crops without considering its residual availability to the following crop. With this objective, a field experiment was carried out to study the direct, residual and cumulative effect of S in a moong–raya rotation on sandy loam soil having 8.2 mg $kg^{-1}$ soil of 0.15% calcium chloride-extractable S. The treatment consisted of four levels of S (0, 10, 20 and 40 kg $ha^{-1}$) applied as gypsum. A significant increase in the grain yield of moong was observed at and above 20 kg S $ha^{-1}$, but the difference between the grain yields at application rates of 20 and 40 kg S $ha^{-1}$ was found to be non-significant. The direct application of 20 kg S $ha^{-1}$ resulted in a significant increase in the grain yield of raya. The residual effect emanating from the application of 40 kg S $ha^{-1}$ to the first crop of moong significantly increased the grain yield of raya. The cumulative application of S at different rates, to both the crops, was not found to be beneficial. It is therefore suggested from this study that the application of 20 kg S $ha^{-1}$ to each crop or 40 kg S $ha^{-1}$ to the first crop of moong was sufficient to obtain optimum yields of both the crops in a moong–raya cropping sequence. The critical levels of S in the whole shoot in moong and raya plants were found to be 0.23 and 0.37%, respectively.

**Key words**: moong, raya, sulphur, direct, residual and cumulative effects

## Introduction

A review of the literature revealed that most studies have been focused on the response of oilseed and pulse crops to S individually (Pasricha and Aulakh, 1991), while reports on the S requirements of cropping systems are rather limited (Sharma et al., 1991). In Punjab, due to the dominance of the rice–wheat cropping system, the cultivation of pulse (moong) and oilseed (raya) crops has largely been restricted to relatively less productive sandy soils, which are expected to be deficient in S. Modern agriculture based on high crop yields, intensive cropping, improved crop varieties and the use of high analysis fertilizer

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is creating a deficit between the addition and removal of sulphur and has further aggravated the situation on these soils, resulting in low yields. In view of the urgent need for diversification, it has become important to encourage the cultivation of these alternative crops in order to help shift some areas from the present rice–wheat cropping system to a pulse–oilseed-based cropping system. States such as Punjab can ill afford to continue with the existing acreage under paddy, which is a great drain on underground water, energy and soil health. In addition under the present-day intensive agricultural conditions, it is increasingly important to calculate the S requirements of the cropping system rather than individual crops in order to account for the carry-over effects and to increase efficiency by rationalizing application rates and thus bring down costs. It is thus desirable to determine the optimum rate and frequency of S application by studying the direct, residual and cumulative effects in a moong–raya cropping system.

## Materials and methods

A field experiment involving a moong–raya rotation was carried out on a S-deficient sandy loam soil with pH 8.2, electrical conductivity 0.30 dS $m^{-1}$ (1:2 soil water suspension) and organic carbon 0.28%. The available phosphorus and potassium in the soil were 14.2 and 128.0 kg $ha^{-1}$, respectively. Soil samples were analysed for initial available Zn, Cu, Fe and Mn by extraction with 0.005 M DTPA buffer solution (diethylene triamine penta-acetic acid containing 0.1 M triethanol amine and 0.01 M $CaCl_2$ adjusted to pH 7.3 with distilled HCl) using a soil : solution ratio of 1:2 and a shaking time of 2 hours (Lindsay and Norvell, 1978). The contents of DTPA-extractable Zn, Cu, Fe and Mn and 0.15% calcium chloride-extractable S were 1.8, 0.8, 7.8, 6.1 and 8.2 mg $kg^{-1}$ soil, respectively. The treatment consisted of four levels of S (0, 10, 20 and 40 kg $ha^{-1}$) applied as gypsum. These treatments were tested for their direct effect on moong and their residual and cumulative effects on raya.

For moong, each treatment was replicated in six plots using a randomized block design (RBD). For the following crop of raya, S treatments were given to three replicates to study the cumulative effect, while the remaining three were kept untreated to study the residual effect of S applied to the previous crop. For the direct effect of S on raya, a further experiment was conducted on the same soil on an adjacent plot of land with all the above sulphur levels. The recommended doses of N, $P_2O_5$ and $K_2O$ were added to both the moong and raya crops. In order to identify the best indicator plant organ for S, whole shoot samples were taken at 45 days of growth for moong and raya from treatments receiving only directly applied S, as per the procedure outlined by Harper and Berkenkamp (1975). Grain and straw samples were taken at maturity. They were washed successively with 0.01 N HCl, tap water, distilled water and de-ionized water. The samples were first dried in air and then at a temperature of 60–70°C in a hot air oven. The dried samples were weighed and ground (pulverized) in a stainless steel grinder, then digested in a 4:1 mixture of $HNO_3$ : $HClO_4$ and analysed for total S using the turbidimetric method (Chesnin and Yien, 1950) and for micronutrients (Zn, Cu, Fe and Mn) in an atomic absorption spectrophotometer. Organic carbon, pH and available P and K were determined by standard methods (Page, 1982). The soil texture was determined by the hydrometer method (Sur and Singh 1976)

## Results and discussion

*Direct effect of S on the yield of moong*

The grain yield of the moong crop increased successively when the level of S was raised from 10 to 40 kg S $ha^{-1}$ (Table 1). This was expected because of the low content of available sulphur in the soil. The increase in grain yield was 147, 256 and 285 kg $ha^{-1}$ over the control treatment at 10, 20 and 40 kg S $ha^{-1}$, respectively. This increase was significant at and above 20 kg S $ha^{-1}$, but the difference between the grain yields at application rates of 20 and 40 kg S $ha^{-1}$ was found to be non-significant. The maximum yield of 901 kg $ha^{-1}$ was obtained when 40 kg S $ha^{-1}$ was applied to the soil. The advantageous effect of S application on lentil has also been reported by Khurana et al. (2002). Kamat et al. (1981) also observed an increase of 510 kg $ha^{-1}$ in green gram with the application of 20 kg S $ha^{-1}$. Mehta and Singh (1979) noted a substantial increase in the grain yield of green gram when S was applied as gypsum or as elemental S on an alkaline calcareous soil in Rajasthan.

The straw yield of moong also increased significantly over the control at all the levels of applied S except at 10 kg S $ha^{-1}$ (Table 2). The application of 10, 20 and 40 kg S $ha^{-1}$ enhanced the straw yield by 4.2, 10 and 11.3%, respectively, over the control.

*Table 1*
Effect of different rates of S (kg $ha^{-1}$) on the grain yield (kg $ha^{-1}$) of moong and raya crops

| Rates of S | Moong grain yield | Raya grain yield | | |
|---|---|---|---|---|
| | Direct | Direct | Residual | Cumulative |
| 0 | 616 | 809 | 767 | 830 |
| 10 | 763 | 830 | 848 | 909 |
| 20 | 872 | 1026 | 960 | 1080 |
| 40 | 901 | 1180 | 1100 | 1183 |
| CD (5%) | 172 | 198 | 221 | 188 |

*Table 2*
Effect of different rates of S (kg $ha^{-1}$) on the straw yield (kg $ha^{-1}$) of moong and raya crops

| Rates of S | Moong straw yield | Raya straw yield | | |
|---|---|---|---|---|
| | Direct | Direct | Residual | Cumulative |
| 0 | 4469 | 4809 | 4618 | 4780 |
| 10 | 4660 | 4920 | 4740 | 4973 |
| 20 | 4916 | 5140 | 5006 | 5164 |
| 40 | 4974 | 5180 | 5103 | 5310 |
| CD (5%) | 285 | 290 | 430 | 280 |

*S content and uptake*

There was a significant increase in the S content in the whole shoot, grain and straw of moong when the level of S was raised from 0 to 40 kg S $ha^{-1}$. The mean sulphur content in the grain at 0, 10, 20 and 40 kg S $ha^{-1}$ was 0.20, 0.28, 0.40 and 0.51%, respectively (Table 3). Similarly, the S content in the straw was 0.07% in the control treatment, increasing significantly to 0.14, 0.21 and 0.23%, respectively, with the application of 10, 20 and 40 kg S $ha^{-1}$ (Table 3). The sulphur content in the whole shoot at 45 days of growth also exhibited an increase with S application.

A perusal of the data in Table 3 revealed that the sulphur uptake by both the grain and straw of moong showed an increasing trend over the control with the application of S at all the rates, with significant increases of 0.96, 2.30 and 3.46 kg S $ha^{-1}$ over the control after the application of 10, 20 and 40 kg S $ha^{-1}$. The sulphur uptake by the straw and the total uptake showed a similar pattern of increase with increasing rates of S application. The increased uptake of sulphur by the moong crop was a consequence of the increase in both the yield and the concentration of S as a result of S application.

*Direct effect of S on the yield of raya*

The application of S at all the rates increased the grain yield of raya, but a significant response was first observed at a rate of 20 kg S $ha^{-1}$. The increase in the grain yield of raya at 20 and 40 kg S $ha^{-1}$ was 217 and 371 kg $ha^{-1}$ over the control. However, the increase observed at 40 kg S $ha^{-1}$ was not significantly higher than that recorded at 20 kg S $ha^{-1}$. The results indicated that an application rate of 10 kg S $ha^{-1}$ was not adequate to cause a significant increase in the grain yield of raya. The straw yield of raya followed the same pattern, increasing significantly from 4809 kg $ha^{-1}$ in the control treatment to 5140 kg $ha^{-1}$ with the application of 20 kg S $ha^{-1}$. The straw yield at 40 kg $ha^{-1}$ of applied sulphur was not significantly higher than that at 20 kg S $ha^{-1}$ (Table 2).

*Residual effect of S on the grain and straw yield of raya*

The application of S to the moong crop built up the available S status in the soil to varying levels depending upon the rate of S application. The amounts of 0.15% $CaCl_2$-extractable S left in the soil after the harvest of moong in the control and in treatments which received 10, 20 and 40 kg S $ha^{-1}$ were 6.2, 7.8, 12.6 and 21.4 mg $kg^{-1}$ soil, respectively. Although the amount of S remaining in the soil after the application of 20 kg S $ha^{-1}$ was above the critical deficiency level of 10 mg $kg^{-1}$ soil, this level was unable to raise the grain yield of raya significantly. However, the residual effect of the application of 40 kg S $ha^{-1}$ provided sufficient sulphur to significantly increase the grain yield of raya (Table 1). Thus, there is a need to redefine the critical deficiency level of S, particularly in crops having a high sulphur requirement, such as brassicas. The

grain yield of raya recorded in the absence of S application was 767 kg $ha^{-1}$, increasing significantly to 1100 kg $ha^{-1}$ with the application of 40 kg S $ha^{-1}$, which was the maximum yield. The same pattern of increase was observed for the straw yield of raya (Table 2). Aulakh and Pasricha (1979) obtained almost double the grain yield for the following green gram crop as the residual effect of 40 kg S $ha^{-1}$ applied either to chickpea or lentil. Biswas and Tewatia (1991) reported spectacular direct and residual responses to S fertilization in various cropping systems. In a groundnut-based system, on the alluvial soils of Kanpur, wheat following groundnut benefited more from residual S (22% yield increase) compared to wheat after rice, where the yield increase was 7% (Tiwari, 1989). In a groundnut–wheat system on the alluvial soils of Punjab, marked direct and residual effects were registered irrespective of the crop fertilized (Aulakh and Pasricha, 1986).

*Cumulative effect of different treatments on the grain and straw yield of raya*

The application of sulphur at different rates to both the crops (moong and raya) was not found to be agronomically beneficial in terms of the grain and straw yields of raya. It can be seen from Table 1 that the application of 20 kg S $ha^{-1}$ to both the crops (cumulative mode) was able to register a significant increase in the grain and straw yield of raya. A perusal of the data in Table 1 indicated that the grain yield of raya (1026 kg $ha^{-1}$) recorded with the direct application of 20 kg S $ha^{-1}$ was comparable to that recorded when 20 kg S $ha^{-1}$ was applied to both the crops (1080 kg $ha^{-1}$). It is therefore suggested that significantly higher yields could be achieved through the application of 20 kg S $ha^{-1}$ to each crop or by applying 40 kg $ha^{-1}$ to the first crop of moong, the residual effect of which was sufficient to satisfy the sulphur requirements of the raya crop.

*Sulphur content in grain and straw*

Data regarding the S content in the grain and straw of the raya crop as influenced by different rates and modes (direct, residual and cumulative) of S application are presented in Table 4. In all three modes, the application of sulphur at different rates enhanced the content of sulphur in both the grain and straw of raya. As expected, a comparatively higher amount of S in the grain and straw was found in the cumulative mode of application, followed by the direct and residual modes of application. In the control, the S content in the grain of raya was 0.27, 0.26 and 0.29% in the direct, residual and cumulative modes of application, increasing to 0.89, 0.78 and 0.94% with the application of 40 kg $ha^{-1}$. The concentration of S in the whole shoot also increased from 0.20% in the control treatment to 0.22, 0.30 and 0.42% at 10, 20 and 40 kg S $ha^{-1}$ under the direct mode of S application.

*Table 3*
Effect of different rates of S on the S content and S uptake of moong

| Rates of S (kg ha$^{-1}$) | S content (%) | | | S uptake (kg ha$^{-1}$) | | |
|---|---|---|---|---|---|---|
| | Whole shoot | Grain | Straw | Grain | Straw | Total |
| 0 | 0.11 | 0.20 | 0.07 | 1.20 | 3.01 | 4.21 |
| 10 | 0.18 | 0.28 | 0.14 | 2.16 | 5.64 | 7.80 |
| 20 | 0.25 | 0.40 | 0.21 | 3.50 | 10.70 | 14.20 |
| 40 | 0.27 | 0.51 | 0.23 | 4.60 | 11.59 | 16.25 |
| CD (5%) | 0.04 | 0.09 | 0.08 | 0.97 | 2.26 | 0.97 |

*Table 4*
Effect of different rates and modes of S application on the S content in raya

| Rates of S (kg ha$^{-1}$) | Whole shoot | S content (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Grain | | | Straw | | |
| | D | D | R | C | D | R | C |
| 0 | 0.20 | 0.27 | 0.26 | 0.29 | 0.11 | 0.09 | 0.12 |
| 10 | 0.22 | 0.35 | 0.32 | 0.42 | 0.14 | 0.11 | 0.15 |
| 20 | 0.30 | 0.62 | 0.58 | 0.68 | 0.18 | 0.16 | 0.20 |
| 40 | 0.42 | 0.89 | 0.78 | 0.94 | 0.22 | 0.20 | 0.24 |
| CD (5%) | 0.04 | 0.15 | 0.10 | 0.06 | NS | NS | NS |

D–Direct, R–Residual, C–Cumulative, NS - Significant

*Indicator plant organ*

The concentration of S in various plant organs can be used as an indicator of the S nutrition status of the plants. In order to find the best indicator organ, coefficients of determination were calculated between the grain yield and the S concentration in the whole shoot, grain and straw in both the moong and raya crops ($R_1$ for moong and $R_2$ for raya). The grain yield had the highest coefficient of determination ($R_1$=0.72; $R_2$=0.83) with the S concentration in the whole shoot, followed by that in the grain ($R_1$=0.64; $R_2$=0.74) and straw ($R_1$=0.55; $R_2$=0.49) in both the crops. This suggested that the whole shoot (45 days of growth) could be used as an indicator of the sulphur status of the plants in both the crops.

*Critical values*

Critical values for the total sulphur content in plants are derived from the yield-composition curve at some arbitrary value, usually 90 or 80% of the maximum. To find the critical levels, regression equations were calculated, where the percentage values of the maximum grain yield of the moong/raya crop were regressed with the corresponding tissue S concentration in the whole shoot, grain and straw. The critical deficiency levels of S were then estimated from these equations at 80% of the maximum. The critical levels of S in moong was found to be 0.23, 0.38 and 0.19% in whole shoot, grain and straw, whereas the corresponding values in raya were 0.37, 0.72 and 0.21%. The data revealed a

lower grain yield when the S content in the whole shoot in moong and raya was below 0.23 and 0.37%, compared to cases when it exceeded these values. This suggested that moong and raya crops should be adequately fertilized, so that the sulphur concentration in the whole shoot should not fall below the respective critical values. Bahl et al. (1986) reported the critical deficiency level of S to be 0.30% and 0.175% at 30 and 60 days of plant growth, respectively, in ground nut.

*Sulphur uptake*

Both the total uptake of S and uptake by the grain and straw increased with increasing rates of S application in all the three modes. A perusal of the data in Table 5 showed that the cumulative mode of S application gave the highest grain, straw and total uptake of sulphur by raya at all rates of application. The S uptake in the direct, residual and cumulative modes of application was 7.49, 6.12 and 8.11 kg $ha^{-1}$, respectively, which increased to 15.74, 13.34 and 17.73 kg $ha^{-1}$ with 20 kg $ha^{-1}$ of applied sulphur. When the total uptake was averaged over the S rates, it was found to be 13.53, 11.55 and 15.28 kg $ha^{-1}$ in the direct, residual and cumulative modes of application.

*Effect of S on micronutrient content in moong and raya grain*

The application of 20 kg S $ha^{-1}$ to S-deficient soil significantly increased the concentration of Zn, Fe, Mn and Cu in moong grain. The zinc content increased significantly from 26.3 μg $g^{-1}$ dry matter in the control to 33.6 and 36.0 μg $g^{-1}$ with the application of 20 and 40 kg S $ha^{-1}$. The iron content improved from 125.3 μg $g^{-1}$ to 140.5 μg $g^{-1}$ with the application of 40 kg S $ha^{-1}$, and significantly higher amounts of Mn (28.4 μg $g^{-1}$ dry matter) and Cu (18.6 μg $g^{-1}$ dry matter) were also present in moong grain at 40 kg S $ha^{-1}$ compared to the control (Table 6).

The direct and cumulative application of S significantly augmented the concentration of Zn, Fe and Mn in raya grain at 40 and 20 kg $ha^{-1}$ applied S, respectively, but not that of Cu (Table 7). It is pertinent to note here that the residual effect of applied sulphur did not improve the concentration of any of the micronutrients. Thus, the application of S, besides increasing the concentration of S, also raised the micronutrient availability to the plants. These results are in accord with the work of Gangadhara et al. (1990) and Khurana et al. (1998), who reported a similar relationship between Zn and S in sunflower and mustard. Bahl et al. (1986) recorded an appreciable increase in the zinc content and uptake by groundnut with the application of increasing levels of sulphur. These results also confirm the findings of Manchanda et al. (1993), who noted that S application increased the content of Zn, Cu, Fe and Mn in lentil. It may be assumed that the direct and cumulative addition of 40 and 20 kg S $ha^{-1}$ resulted in a decline in the soil pH in the rhizosphere, leading to higher micronutrient concentrations. It is also possible that the applied S might have helped the roots to proliferate into deeper soil layers, thus allowing the plants to tap the nutrients from these layers.

*Table 5*
Effect of different rates and modes of S application on S uptake by raya

| Rates of S (kg ha$^{-1}$) | S uptake (kg ha$^{-1}$) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Grain | | | Straw | | | Total | | |
| | D | R | C | D | R | C | D | R | C |
| 0 | 2.23 | 1.97 | 2.41 | 5.26 | 4.15 | 5.70 | 7.49 | 6.12 | 8.11 |
| 10 | 2.95 | 2.70 | 3.85 | 6.91 | 5.69 | 7.36 | 9.86 | 7.96 | 11.31 |
| 20 | 6.45 | 5.83 | 7.38 | 9.29 | 8.32 | 10.34 | 15.74 | 13.34 | 17.73 |
| 40 | 10.54 | 8.51 | 11.23 | 11.39 | 10.22 | 12.86 | 21.93 | 18.79 | 24.09 |
| CD (5%) | 2.80 | 1.26 | 1.74 | 3.99 | 4.10 | 4.70 | 3.43 | 5.84 | 4.76 |

D–Direct, R–Residual, C–Cumulative,

*Table 6*
Direct effect of sulphur on the micronutrient content in moong grain

| Rates of S (kg ha$^{-1}$) | Content (μg g$^{-1}$) | | | |
|---|---|---|---|---|
| | Zinc | Manganese | Iron | Copper |
| 0 | 26.3 | 23.8 | 125.3 | 14.7 |
| 10 | 28.7 | 25.4 | 132.5 | 16.03 |
| 20 | 33.6 | 27.2 | 137.8 | 17.7 |
| 40 | 36.0 | 28.4 | 140.5 | 18.6 |
| CD (5%) | 2.83 | 1.95 | 4.43 | 1.61 |

*Table 7*
Direct, residual and cumulative effect of sulphur on the micronutrient content in raya grain

| Rates of S (kg ha$^{-1}$) | Content (μg g$^{-1}$) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Zn | | | Mn | | | Fe | | | Cu | | |
| | D | R | C | D | R | C | D | R | C | D | R | C |
| 0 | 44.2 | 44.0 | 44.2 | 35.2 | 34.2 | 34.0 | 102.3 | 100.3 | 104.0 | 18.1 | 17.7 | 18.1 |
| 10 | 45.2 | 45.9 | 45.8 | 36.1 | 34.6 | 36.2 | 105.1 | 101.3 | 106.3 | 18.4 | 17.9 | 18.9 |
| 20 | 47.1 | 46.4 | 48.4 | 37.7 | 35.7 | 40.4 | 109.3 | 103.0 | 113.6 | 18.8 | 18.2 | 19.2 |
| 40 | 50.2 | 46.8 | 51.7 | 41.5 | 35.6 | 41.8 | 110.6 | 106.6 | 118.0 | 18.9 | 18.3 | 20.0 |
| CD (5%) | 3.5 | NS | 2.3 | 3.0 | NS | 2.7 | 3.6 | NS | 7.3 | NS | NS | NS |

D–Direct, R–Residual, C–Cumulative, NS – Non-significant

## Conclusions

In a moong–raya cropping system, the direct application of S at a rate of 20 kg ha$^{-1}$ individually to moong and raya significantly increased their grain yields. However, the application of 40 kg ha$^{-1}$ to the first crop of moong also gave optimum yields of both the crops, suggesting that a portion of the applied S remained in the soil, where it significantly increased the grain yield and S uptake in the subsequent raya crop. The cumulative mode of S application at different rates was not found to be remunerative in terms of grain and straw yields, and appeared to be a wasteful investment. It was further concluded from this study that the residual effect of S should be considered in order to rationalize the rates of application and to bring down the costs of cultivation.

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# CORRELATION BETWEEN MAIZE GENOTYPES AND THE STALK ROT CAUSED BY MAIZE *Fusarium*

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Six single-cross hybrids and their parental lines were inoculated with the FG36 *Fusarium graminearum* isolate in 2005 and 2006. In both years the degree of infection increased after artificial inoculation for both the hybrids and the inbred lines compared with the level of natural infection. The more severe stalk infection recorded in 2005 than in 2006 could be attributed to the weather conditions during flowering and harvesting.

The 18 genotypes examined exhibited different levels of resistance to fusarium stalk rot. It could be concluded from the results that the resistance level of the female parent was decisive in the inheritance of the response to fusarium stalk rot (female component–hybrid $r = 0.88$, male component–hybrid $r = 0.39$).

Some genotypes may be severely affected in epidemic years, while exhibiting a lower rate of infection in years with lower pathogen pressure. This suggests that successful breeding for resistance can only be carried out efficiently by means of artificial inoculation.

**Key words:** maize, stalk rot, *Fusarium graminearum*, resistance

## Introduction

The stalk strength of maize is of outstanding importance for reliable maize production. Stalk strength depends on two major factors: the mechanical structure of the stalk and the occurrence of stalk rot caused by various *Fusarium* and *Macrophomina* species. Thanks to constant efforts by breeders, the mechanical stalk strength properties of currently cultivated hybrids are satisfactory, but this is not always true of the resistance of the hybrids to fusarium stalk rot. This is partly because breeders select mainly on the basis of natural infection, which is not always sufficient.

Stalk rot is a pathological process developing as the consequence of colonisation by various pathogens. The mass appearance of stalk rot begins

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when the ears reach physiological maturity. Surveys carried out in Hungary and abroad have indicated that *Fusarium* species are the primary and most frequently occurring pathogens responsible for this disease (Krüger, 1985; Kizmus et al., 2000). The disease leads to dry rot in the stalk, caused mainly by enzymes produced by the *Fusarium* pathogen, which decompose the cell-wall (Szécsi, 1975; Lorenzo et al., 1997; Szőke et al., 2006). As the result of infection, the plants have far lower yields than their healthy counterparts. The yield losses resulting from this damage may be as great as 13–20% (Jugenheimer, 1940).

The level of infection is greatly influenced by environmental factors, the genotype × environment interaction and the pathogen resistance of the given maize genotype. Considerable differences in *Fusarium* stalk rot resistance have been observed between maize hybrids and inbred lines (Jugenheimer, 1940; Mesterházy, 1981; Kovács et al., 1988; Ledencan et al., 2003).

## Materials and methods

The aim was to determine the resistance of Martonvásár maize genotypes to *Fusarium* stalk rot. Eighteen genotypes (six single-cross hybrids and their parental lines) were inoculated with the FG36 isolate of *Fusarium graminearum* in 2005 and 2006. This isolate was selected in preliminary phytotron tests to find one of the most aggressive of ten different isolates. Inoculation was carried out by placing infected wheat grains in the second internode from the base of the plant. Following size grading, the wheat grains were sterilised and placed in test-tubes, 100 to a tube, after which 2 ml of a $10^6$ conidia/ml suspension of the FG36 isolate was added to each tube. Inoculated grains were then placed in holes made in the stalk using a 2 mm hand drill. Sterile wheat grains were used as a control. The holes were then sealed with sticking plaster to prevent natural infection. The tested genotypes were sown in a split-plot design with four replications, with the genotypes in the main plots and the treatments in the sub-plots. Sampling and processing began on 29 October. The stalk samples were cut in half lengthwise and the length and width of the lesions visible on the pith were measured. The area of the lesions was calculated as $\pi \times a \times b$ ($cm^2$). The data were evaluated using analysis of variance (Sváb, 1981).

## Results and discussion

As a result of artificial inoculation both the hybrids and the lines suffered a substantial level of infection in both seasons. As a percentage of the control, the hybrids exhibited a higher level of infection than the inbred lines (68.10%). At first glance this is surprising, as a lower level of infection might be expected due to the heterosis effect. One explanation could be that the genotypes investigated had different vegetation periods, those of the lines generally being longer, which meant that they were exposed to the effect of artificial inoculation for a longer period. In addition, as the internodes of the lines were considerably shorter than those of the hybrids, the relative values obtained were smaller. This fact will be taken into account in future research.

Of the two experimental years, a higher rate of infection was observed in 2005 for both the hybrids and the lines (by 110.6% and 54.8%, respectively).

This can be attributed to meteorological factors. The data for the two years indicated that infection was enhanced by a lower quantity of rainfall during flowering and a wetter autumn (Ullstrup, 1955). An investigation of the infection × year (B×C) interaction revealed that the hybrids suffered a higher rate of infection than the lines in both years, while the difference in infection level between the hybrids and lines was similar in the two years (Fig. 1).

With the exception of the infection × year (B×C) interaction for the hybrids (P=10%), all the factors analysed in the experiment had a significant effect at the 5% level of probability (Table 1). It is clear from the mean square (MS) values that in the two years of the experiment the factors genotype and infection (A, B) had the greatest influence on the size of the lesion. In most cases the interactions were also significant, but their variance was an order of magnitude smaller than that of the main effects.

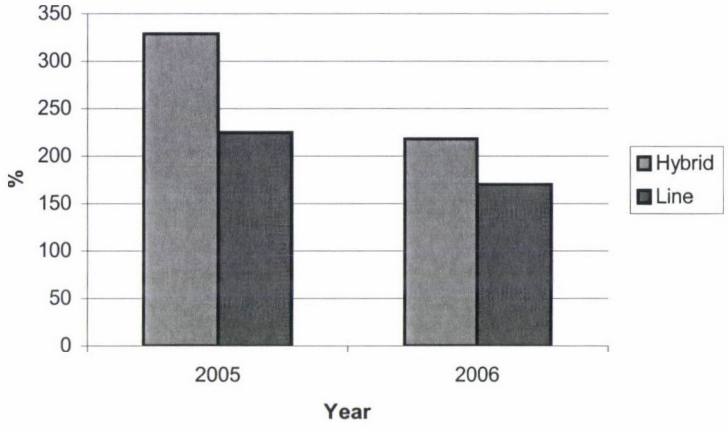



*Fig. 1.* Mean stalk rot reactions of hybrids and their parental lines as a percentage of the control

*Table 1*
Analysis of variance on the size of the lesions

| Factor | Hybrid | | Line | |
|---|---|---|---|---|
| | MS | | | |
| | FG | Size of lesion, $cm^2$ | FG | Size of lesion, $cm^2$ |
| Genotype (A) | 5 | 3526.77* | 11 | 645.068* |
| Infection (B) | 1 | 23179.775* | 1 | 8209.008* |
| Year (C) | 1 | 700.002* | 1 | 255.256* |
| (A×B) | 5 | 841.118* | 11 | 189.873* |
| (A×C) | 5 | 577.601* | 11 | 300.254* |
| (B×C) | 1 | 242.666+ | 1 | 160.016* |
| (A×B×C) | 5 | 173.716* | 11 | 84.244* |
| Error | 69 | 67.761 | 141 | 23.09 |

The relative level of infection recorded for the six hybrids and twelve parental lines is illustrated in Figure 2 compared to the experimental mean (100%). Substantial differences were observed between the maize genotypes, particularly in the case of the inbred lines, six of which exhibited above-average infection. Two of these (C, G) were used as female parent, and four (B, C, H, L) as male component in the hybrids. Among the female components the lowest rate of infection was found for lines K and A.

Two of the hybrids were found to exhibit above-average infection (MV2 and MV4) and in both cases the female component also became severely infected. Although line L, the most infected male parent, exhibited far greater infection than the inbred line mean, hybrid MV6 had the second lowest rate of infection among the hybrids. Hybrids MV1 and MV3 exhibited below-average infection, and in both cases the female component had a lower rate of infection than the male line. The lowest level of infection was recorded for hybrid MV5, the only hybrid where the female component exhibited greater infection than the male parent. The relative levels of infection after artificial inoculation for the female and male parents and for the hybrids were used to calculate correlation coefficients between the parents and hybrids. The correlation was found to be $r = 0.88$ for the female parent and $r = 0.39$ for the male parent. Similar values were obtained by Georgiev (1977) and Marton (2002). These results suggest that the inheritance of *Fusarium* stalk rot resistance is more closely correlated with the resistance of the female parent.

Figure 3 depicts the increase in the level of infection as the result of artificial inoculation compared with the control. It is clear from the figure that in the control treatment hybrid MV1 exhibited the lowest level of infection, while after artificial inoculation this hybrid responded with the greatest increase in the infection level (440%). Without inoculation hybrid MV2 had the highest level of infection, while the increase due to inoculation was only around 200%. The rate of infection in the control treatment was low for hybrid MV5 and was not substantially increased by inoculation.

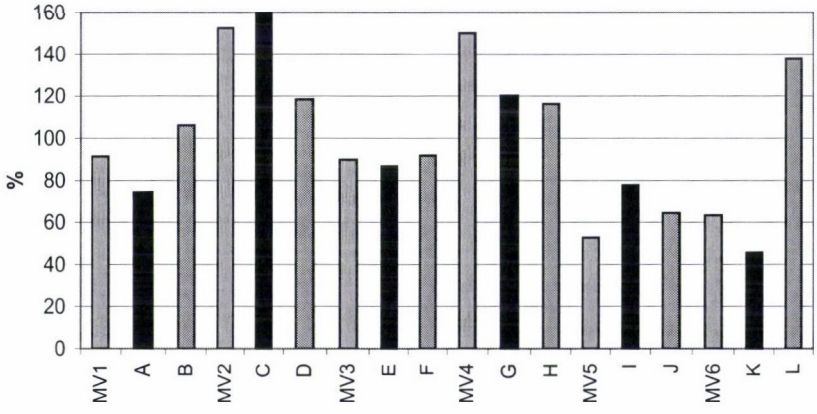



*Fig. 2.* Level of *Fusarium* stalk infection relative to the experimental mean (100%) for the hybrids (striped) and their female (black) and male (hatched) parental lines

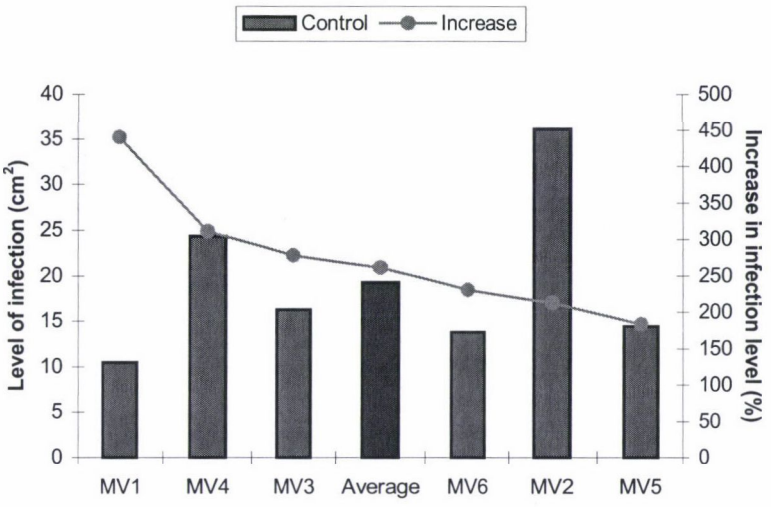



*Fig. 3.* Increase in the level of *Fusarium* stalk infection in hybrids due to artificial inoculation compared with the control

These results prove that successful selection cannot be based only on a knowledge of natural infection data, as the characteristics of a genotype may change to a great extent in the case of strong pathogen pressure (e.g. in epidemic years), as observed for hybrids MV1, MV3 and MV6.

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# SOIL CHANGES AFTER FORTY YEARS OF SUCCESSION IN AN ABANDONED COPPICE IN THE CZECH REPUBLIC

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Soil processes over forty years of woodland succession were studied in the abandoned coppices of the Děvín Nature Reserve, in the south-east of the Czech Republic. A total of 113 horizon samples from 34 profiles were taken in the 1960s and 2000s, following identical field and laboratory approaches, to characterize soil texture, contents of carbonates and organic matter, and soil reaction (pH/$H_2O$, pH/KCl). Changes in the soil properties were discussed in relation to the gradual development of the mature woodland that replaced the former intensively managed ancient coppice. Four soil types (Luvisols, Regosols, Leptosols and Chernozems) and their horizons were statistically treated to identify distributions/shifts in the measured values from the past to the present. The following results were obtained: (1) The horizontal transport and sedimentation of sandy calcareous particles into the Leptosols topsoil led to increased acidity. (2) In Luvisols, the same was detected for fine clayey particles. This can be explained by the topographical occurrence of the two types – on the upper parts of slopes and under limestone cliffs for the former, and in the flat foothills for the latter soil type. (3) No acidification appeared except for Luvisols, whose luvic horizons E and Bt are, in contrast to the others, poor in calcium carbonate and relatively acidic. A decrease in acidity was recorded in KCl solution, but not in $H_2O$. This is interpreted as the consequence of the buffering ability of the soil sorption complex. (4) Slightly improved humification was only detected in the surface horizons of Luvisols and Leptosols. (5) Contrary to expectations no illimerization, i.e. the migration of clay particles from topsoil to subsoil, was revealed.

As forty years is apparently too short a time for significant vertical clay migration, it was concluded that i) horizontal migration and the accumulation of substrate particles was of at least the same importance as *in situ* pedogenetic processes, and ii) soil property dynamics that could be linked with the changed woodland management were proved to act relatively slowly.

**Key words**: soil change, coppice, succession

## Introduction

Coppices are an ancient management form of woodlands, widespread in Europe (Billamboz, 2003; Coch, 2003; Rackham, 2003; Szabó, 2005). Traditional coppice management involves a regular short-rotation cutting cycle

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and other practices such as litter raking and occasional wood pasture (Fuller and Peterken, 1996; Matthews, 2001). In Central European countries, coppicing was gradually abandoned at the latest by the mid-20$^{th}$ century. By this time, most coppices were singled out and allowed to grow as a high forest (Matthews, 2001). Woodland succession took over the main role in the shaping of the ecosystem.

The present study deals with long-term soil changes in the woodlands of Děvín, probably the best-preserved over-aged coppice in the Czech Republic. The establishment of a nature reserve in 1949 largely prevented the traditional management activities, especially coppice management, and counteracted the influence of litter raking (Hofmeister et al., 2002). Shady forest conditions began to develop. As the nature reserve was combined with a game preserve (Danihelka, 2003; Heroldová, 2003) the high concentration of ungulates led to mechanical damage to the vegetation and to eutrophication (Chytrý and Danihelka, 1993; Reimoser and Gossow, 1996), so in 1996, mouflon and red deer were removed from the area.

The research approach was based on a comparison of past and recent datasets for spatially corresponding sites – soil pits and horizons. Fresh data were compared with those recorded by J. Horák as part of a Czech forest typology survey in the 1950s and 1960s (Horák, 1967; 1969). Such temporal studies are commonly used for investigations of long-term forest dynamics, such as those of Falkengren-Grerup (1986), who studied 34 plots from 1949 to 1970, Billett et al. (1990), who studied changes from 1949 to 1987, Falkengren-Grerup (1990), who presented data on more than a hundred plots studied from 1929 to 1988, and Falkengren-Grerup and Tyler (1991), who studied 95 plots from 1979 to 1990. However, these authors generally focused on the influence of acidification and eutrophication.

The present study aimed to evaluate the changes in five soil properties between 1963–1964 and 2002–2004 in the woodlands of Děvín. Two basic parallel hypotheses were postulated: neutral $H_0$ "No statistically significant differences will be detected in measured parameters and soil categories (types and horizons)", and alternative $H_A$ "Statistically significant differences will be detected in measured parameters and soil categories (types and horizons)". Four soil units were distinguished: Luvisols, Regosols, Leptosols and Chernozems (ISSS-ISRIC-FAO, 1998), including 14 more or less homogeneous types of pedogenetic horizons (see Material and Methods for descriptions). These obviously differ in physical and chemical composition, and have developed under different site conditions. It was therefore necessary to make the hypotheses more specific.

Considering the constitution and pedogenetic processes forming the particular soil types, and assuming changes related to abandonment of light, relatively drier coppice wood and gradual succession to the shady, moister microclimate of a closed forest, ten hypotheses were formulated:

$H_1$: In Luvisols, significant texture changes in luvic horizons: loss of clay in eluvial, and increase of clay in illuvial horizons.

$H_2$: No significant textural changes in other soil types.

$H_3$: No significant changes in other texture fractions.

$H_4$: Significant decrease in content of carbonates in upper soil horizons of Luvisols and Regosols.

$H_5$: No significant change in content of carbonates in other soil types and horizons, namely the subsoil.

$H_6$: Significant increase in organic matter content in the uppermost soil horizons of all soil types.

$H_7$: No change in the organic matter content in mineral soil horizons.

$H_8$: Significant decrease in the soil reaction in Luvisols and Regosols, particularly in the upper horizons.

$H_9$: More intensive decrease in the soil reaction in KCl than in $H_2O$ for Luvisols and Regosols.

$H_{10}$: No significant change in the soil reaction in Leptosols and Chernozems.

The hypotheses cover the major potential changes/steady state that can be identified using the values of the five measured parameters: texture, content of carbonates, content of organic matter, pH in water and pH in KCl suspension. However, soils are very heterogeneous, complex entities, which implies that the detected changes do not necessarily tell us the complete story. The obvious limitation is the set of parameters selected 50 years ago, when it could not have been expected that the measurements would be repeated in the future in the search for soil changes.

## Materials and methods

*Study area*

Děvín is the central peak of a small but conspicuous hilly area in the South-Eastern corner of the Czech Republic. With an elevation of 550 m a.s.l. it dominates the landscape, being surrounded by the lowlands and alluvial floodplains of NW Pannonia. Because of its natural and cultural values it became a National Nature Reserve (380 ha) and is one of the cores of the Dolní Morava Biosphere Reserve (Danihelka, 2003). Děvín is a Jurassic limestone cliff with foothills covered with Quaternary loess. The chemically pure limestone originated in the Upper Dogger or Callovian period, 170 million years ago, while the loess sediments are often decalcified. The carbonate content in the soils differs markedly from one place to the other; soils connected to limestone and to loess (sometimes decalcified) can be found close to each other. Roughly three quarters of Děvín's area is covered by forest; the rest is mainly thermophilous grassland, then shrubby and arable land, etc. The forests include a gradient from xerothermic oak-forests (*Quercion pubescenti-petraeae*), through thermophilous to mesophilous oak–hornbeam forests (*Carpinion*), to ravine forests dominated by broad-leaved lime (*Tilio-Acerion*). The forest had been managed for many centuries as coppices, with a cutting period of at most 30 years (usually 10–20 years); in the regular pattern, 100–150-year-old standards of sessile oak were left. The major part of the Děvín woodland preserved its coppice structure until the present (Fig. 1).



*Fig. 1.* Aged coppice wood in Děvín, 2002

*Sampling design*

Horák (1967, 1969) opened and sampled forty-one soil pits, determining the soil types using the classification system of Pelíšek (1964). It proved possible to locate the positions of the pits using Horák's map (scale 1:10,000) showing the position of the sites, and his original field notebooks with descriptions of the investigated spots. New surveys were made on 34 soil pits; the profiles were described and 113 soil samples were taken from the same horizons as Horák. The sampling methodology followed that reported by Catt (1990); the soil profiles and horizons are listed in the Appendix.

*Soil units*

The subrecent pedogenesis in the study area was substantially conditioned by humification (in Holocene), eolic accumulation (Quaternary origin of loess) and slope sedimentation (subrecent gravitative movement of soil masses); for more information on sedimentary substrates in the Czech Republic see Růžičková et al. (2003). Karstified limestones are the major bedrock. Anthropogenic erosion played an important role during the past five to eight thousand years (Hédl, 2005). The soils can be characterized on the basis of field descriptions and laboratory measurements (see Appendix). The soils usually contained 40–50% of particles with a size of <0.01 mm and, except for a few horizons, they were calcareous, humus-rich in the topsoil and neutral to moderately basic.

The soils were basically classified in the field; older (Pelíšek, 1964) and more recent (ISSS-ISRIC-FAO, 1998) soil classification systems could be matched in all cases, though they delimited the recognized units in a narrower or broader sense.

i) Luvisols, 10 profiles. Characterized by clay accumulation and aggregation in Bt-horizons, and eluvial E-horizons, which were analytically detectable by texture differences. Little or no content of carbonates was found, except for the substrate C-horizons, and there was a low content of organic matter, except for the surface A-horizon. Acidity was around neutral or moderate. Haplic Luvisols were common all over the studied area, occurring on the moderately inclined middle and lower parts of the slopes.

ii) Regosols, 7 profiles. Soils originating from sub-recent slope accumulation having A-horizons partly accumulated in historical times, probably by erosion after deforestation, and C-horizons with a low humus content and much lighter colour, partly accumulated by solifluction. The content of carbonates was low to intermediate and soil acidity around neutral to basic. Calcic Regosols occurred on lower parts of the slopes with moderate inclination.

iii) Leptosols, 12 profiles. The soil bodies were developed by intensive humification on weathered limestone having humus- and carbonate-rich A-horizons with a thickness of at least 20 cm. Rendzic, Mollic or Eutric Leptosols were found on the upper parts of slopes with greater inclination.

iv) Chernozems, 5 profiles. The A-horizons were more than 30 cm thick, very dark in colour and contained carbonates. The C-horizon originated from loess materials rich in carbonates, sometimes with an admixture of stones. Haplic Chernozems were quite rare soil types – their persistence until recent times is probably due to continuous agricultural practices (vineyards) at these sites. Slight illimerization under woodland conditions led to their classification as Luvic Chernozems, occurring sparsely in the foothills.

*Statistics*

Statistical analyses were performed using STATISTICA, 6.0 version (StatSoft Inc., 2001). As the datasets did not show normal distribution, non-parametric testing was applied using the Wilcoxon test (Havránek, 1993; Sokal and Rohlf, 1981). Only datasets consisting of at least 5 cases were included; hence, for Chernozems only the textural classes and carbonate contents were considered. The data distribution was visualized by box-and-whisker plots depicting medians, interquartile range, non-outlier range (coefficient 1) and outliers (coefficient 1.5). The comparison was always within pairs of boxplots for old and new values.

For statistical comparison the following fourteen categories were set up (ISSS-ISRIC-FAO, 1998) consisting of fairly homogeneous horizons belonging to particular soil types. The statistical testing of the differences between old (1960s) and new (2000s) values was focused on the following categories:

i) Luvisols
– A: surface Ah organo–mineral horizons
– E: eluvial E, AE and partly transitional AB horizons
– Bt: argilic Bt, Bt1 and Bt2 horizons
– C: substrate Ck, C and partly transitional BC horizons

ii) Regosols
– A1: surface Ah1 organo–mineral horizons
– A2: Ah2 and Ah3 organo–mineral horizons, accumulated by geli– and solifluction
– C: (accumulated) calcareous substrate Ck, Ck1 and Ck2 horizons

iii) Leptosols
– A1: surface Ah1 organo–mineral horizons
– A2: subsurface Ah2 organo–mineral horizons
– C: substrate Ck (calcareous) and C horizons
– C+: as previous, plus transitional Bw and AC horizons

iv) Chernozems
– A1: surface Ah and Ah1 organo–mineral horizons
– A2: subsurface, transitional AC, Ah2 and AB horizons
– C: substrate calcareous Ck horizons

*Laboratory analyses*

In order to obtain data comparable with those provided by Horák (1967; 1969), the original analytical methods were repeated. The following treatments were performed on air-dried, sieved (mesh 2 mm) samples:

i) Weight percentage assessment of texture classes by the elutriation method of Kopecký (1928). Four texture classes were determined (note that the delimitation of fractions may differ from modern systems): i) clay (fraction I – particles with diameter less than 0.01 mm), loam (fraction II – 0.01–0.05 mm), fine sand (fraction III – 0.05–0.1 mm) and sand (fraction IV – 0.1–2.0 mm). The authors used the original laboratory equipment from the 1950s.

ii) Calcium carbonate content by the volumetrical determination of Laník and Halada (1956). The authors used the original laboratory equipment from the 1950s.

iii) Organic matter content determined in a mixture of $K_2Cr_2O_7$ and $H_2SO_4$ by the method of Walkley and Black (1934) modified by Novák (1950).

iv) Determinations of pH in water and KCl by the method of Válek (1954), where air-dried samples were treated in a 1:2.5 solution of $H_2O$:1 M KCl and measured with a glass electrode.

## Results

Data changes in textural classes are given in Table 1, and data on carbonate and organic matter contents and soil reactions in Table 2.

*Table 1*

Summarised results of Wilcoxon's matched pair test for differences in four texture classes, between old (1960s) and new (2000s) samples

| Soil type | Horizon | N | I. Clay | | | II. Loam | | | III. Fine sand | | | IV. Sand | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | +/– | Z | p | +/– | Z | p | +/– | Z | p | +/– | Z | p |
| Luvisols | 1.A | 9 | ++ | 1.36 | 0.173 | | 0.06 | 0.953 | – – | 1.24 | 0.214 | | 0.77 | 0.441 |
| | 2.E | 7 | | 1.52 | 0.128 | – | 1.01 | 0.310 | – | 0.17 | 0.866 | | 0.17 | 0.866 |
| | 3.Bt | 10 | | 0.89 | 0.374 | | 0.53 | 0.594 | | 0.18 | 0.589 | | 1.13 | 0.260 |
| | 4.C | 9 | + | 0.53 | 0.594 | ++ | **2.19** | **0.028** | – – | **2.19** | **0.028** | – | **1.95** | **0.050** |
| Regosols | 1.A1 | 6 | – | 0.73 | 0.463 | – – | 1.36 | 0.173 | + | 1.15 | 0.249 | + | 0.94 | 0.345 |
| | 2.A2 | 10 | – – | **2.19** | **0.028** | + | 1.56 | 0.114 | + | 0.97 | 0.333 | | 0.97 | 0.333 |
| | 3.C | 7 | + | 0.34 | 0.735 | ++ | **2.03** | **0.043** | – | **2.03** | **0.043** | – | **2.20** | **0.023** |
| Leptosols | 1.A1 | 12 | – | 0.46 | 0.650 | – – | **2.40** | **0.017** | + | 1.38 | 0.169 | + | 0.97 | 0.333 |
| | 2.A2 | 7 | – | 1.01 | 0.310 | – | 1.69 | 0.091 | | 0.86 | 0.398 | ++ | 1.86 | 0.063 |
| | 3.C | 13 | | 0.87 | 0.386 | + | **1.99** | **0.047** | | 0.87 | 0.386 | – | **1.99** | **0.047** |
| | 4.C+ | 16 | | 0.87 | 0.386 | + | **1.99** | **0.047** | | 0.87 | 0.386 | – | **1.99** | **0.047** |
| Chernozems | 1.A1 | 5 | ++ | 1.21 | 0.225 | – – | 1.75 | 0.080 | + | 0.40 | 0.686 | | 0.67 | 0.500 |
| | 2.A2 | 6 | + | 1.57 | 0.116 | – | 0.52 | 0.600 | | 0.10 | 0.917 | – – | **2.20** | **0.028** |
| | 3.C | 5 | ++ | 0.94 | 0.345 | | 0.40 | 0.686 | – | 0.94 | 0.345 | | 1.48 | 0.138 |

N: number of cases, +/–: change observed from boxplots; ++: major increase; + increase; – decrease; – – major decrease; Z: test value; p: level of significance. For delimitation of soil horizons see text. Tests with p values significant at the 5% level are marked in bold.

*Table 2*
Summarised results of Wilcoxon's matched pair test for differences in soil parameters, between old (1960s) and new (2000s) samples

| Soil type | Horizon | Carbonates | | | | Org. matter content | | | | pH $H_2O$ | | | | pH KCl | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *N* | +/– | Z | p | *N* | +/– | Z | p | *N* | +/– | Z | p | *N* | +/– | Z | p |
| Luvisols | 1.A | 10 | | 0.34 | 0.735 | 10 | + | 0.56 | 0.575 | 10 | | 0.76 | 0.445 | 10 | – | 1.58 | 0.114 |
| | 2.E | 7 | | 0.00 | 1.000 | 7 | + | 0.85 | 0.398 | 7 | | 0.51 | 0.612 | 7 | – – | 1.86 | 0.063 |
| | 3.Bt | 10 | | 0.67 | 0.500 | 11 | | 0.76 | 0.445 | 11 | + | 1.51 | 0.131 | 11 | – | 1.42 | 0.155 |
| | 4.C | 9 | – – | **2.19** | **0.028** | 10 | | 0.30 | 0.767 | 10 | + | 0.56 | 0.575 | 10 | | 0.66 | 0.508 |
| Regosols | 1.A1 | 6 | – | **1.99** | **0.047** | 6 | – | 1.48 | 0.138 | 7 | | 0.34 | 0.735 | 7 | – | 0.85 | 0.398 |
| | 2.A2 | 10 | – | **1.99** | **0.047** | 10 | + | 0.46 | 0.646 | 10 | + | **2.09** | **0.037** | 10 | + | 1.38 | 0.169 |
| | 3.C | 7 | – | 1.69 | 0.091 | 7 | | 0.85 | 0.398 | 7 | ++ | **2.37** | **0.017** | 7 | | 1.18 | 0.237 |
| Leptosols | 1.A1 | 12 | + | **2.27** | **0.023** | 11 | | 0.97 | 0.333 | 11 | ++ | **2.93** | **0.003** | 11 | + | 1.69 | 0.091 |
| | 2.A2 | 7 | + | 0.34 | 0.735 | 6 | + | 1.15 | 0.249 | 6 | ++ | **2.02** | **0.043** | 6 | + | 1.78 | 0.075 |
| | 3.C | 13 | | 0.78 | 0.433 | 12 | | 1.57 | 0.117 | 12 | ++ | **3.06** | **0.002** | 12 | + | **2.35** | **0.019** |
| | 4.C+ | 16 | | 0.65 | 0.513 | 15 | | 0.11 | 0.091 | 15 | ++ | **3.40** | **0.000** | 15 | + | **2.47** | **0.013** |
| Chernozems | 1.A1 | 5 | | 0.40 | 0.686 | 3 | / | / | / | 3 | / | / | / | 3 | / | / | / |
| | 2.A2 | 6 | + | 0.52 | 0.600 | 4 | / | / | / | 4 | / | / | / | 4 | / | / | / |
| | 3.C | 5 | | 0.00 | 1.000 | 3 | / | / | / | 3 | / | / | / | 3 | / | / | / |

No test was performed for less than five cases (denoted as /). See Table 1 for explanation of symbols and abbreviations. Significant p values are marked as follows: **≤0.01**; **≤0.05**.

*Texture*

The subsoil horizons of all four soil types show an almost uniform loss of coarser fractions (fine sand and sand) and a corresponding gain in finer fractions (clay and loam). However, no such general pattern can be documented in the topsoil. In Luvisols (Fig. 2) and Chernozems (Fig. 5) the fine fractions (mostly I, clay) increased, while in Leptosols (Fig. 4) and Regosols (Fig. 3) the coarse fractions (mostly IV, sand) increased.

The following statistically significant changes were observed:

*i) Clay*: statistically significant ($p < 0.05$) decrease in the A2 horizon of Regosols; perceptible but statistically non-significant ($p > 0.05$) increase in the uppermost horizons of Luvisols and Chernozems and in the subsoil of Chernozems.

*ii) Loam*: statistically significant ($p < 0.05$) increase in the subsoil (C-horizons) of Luvisols, Regosols and Leptosols; decrease in the the uppermost horizon of Leptosols.

*iii) Fine sand*: statistically significant ($p < 0.05$) decrease in subsoil (C-horizons) of Luvisols and Regosols; perceptible but non-significant ($p > 0.05$) decrease in the uppermost horizon of Luvisols.

*iv) Sand*: statistically significant ($p < 0.05$) decrease in the C-horizon of Luvisols, Regosols and Leptosols, and in the A2-horizon of Chernozems.

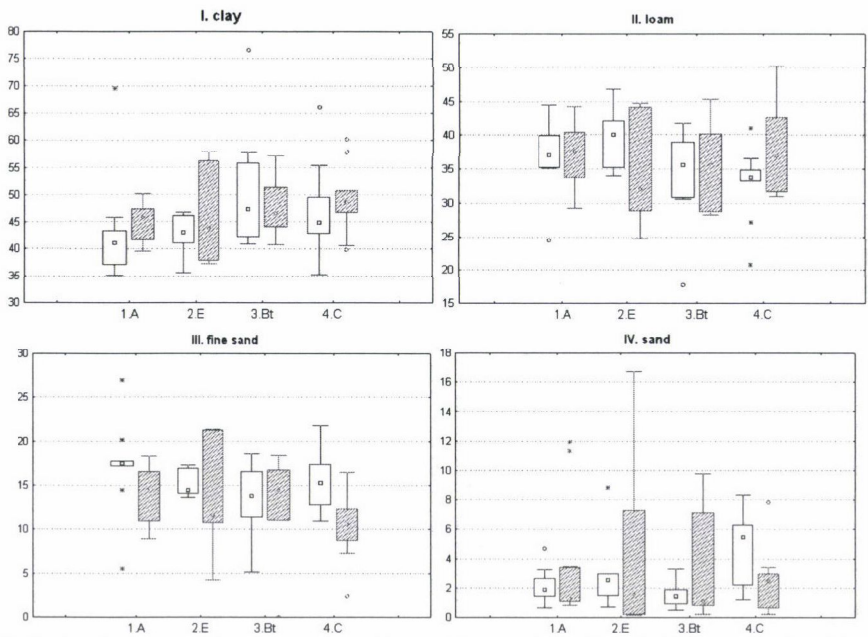



*Fig. 2.* Luvisols, changes in texture, four classes; X-axes show four horizon categories. Boxplots of medians (inside-squares), interquartile range (boxes), non-outlier range (whiskers), and outliers (circles, double-crosses) show old values (1960s) in left-hand white boxes and new values (2000s) in right-hand hatched boxes. The most striking changes are in horizon 4.C, with intermediate changes in the 2.E and 1.A horizons. No change can be observed in horizon 3.B

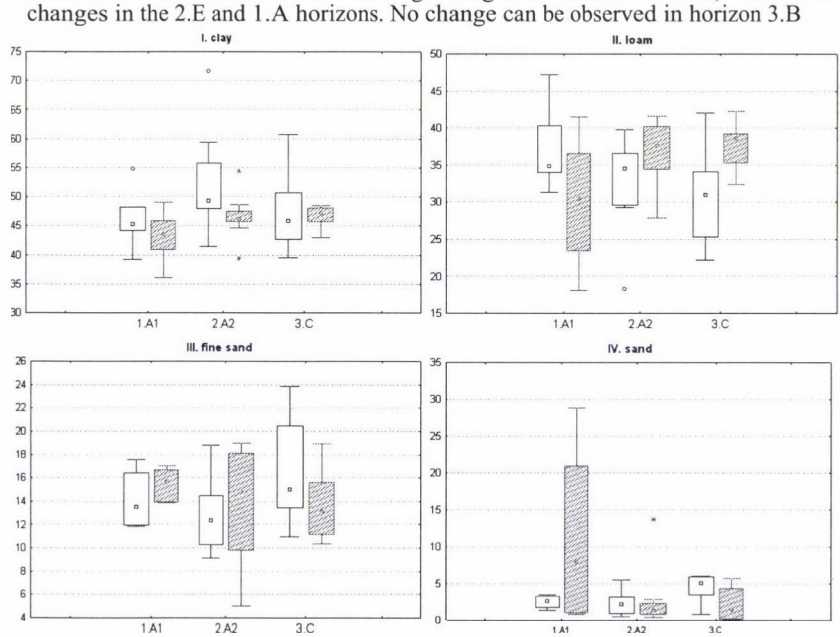



*Fig. 3.* Regosols, changes in texture, four classes; X-axes show three horizon categories. For graph design see Fig. 2. Amounts of most fractions changed; the overall process can be described as loss of finer fractions in the topsoil (1.A, less in 2.A) and their increase in the subsoil (3.C)

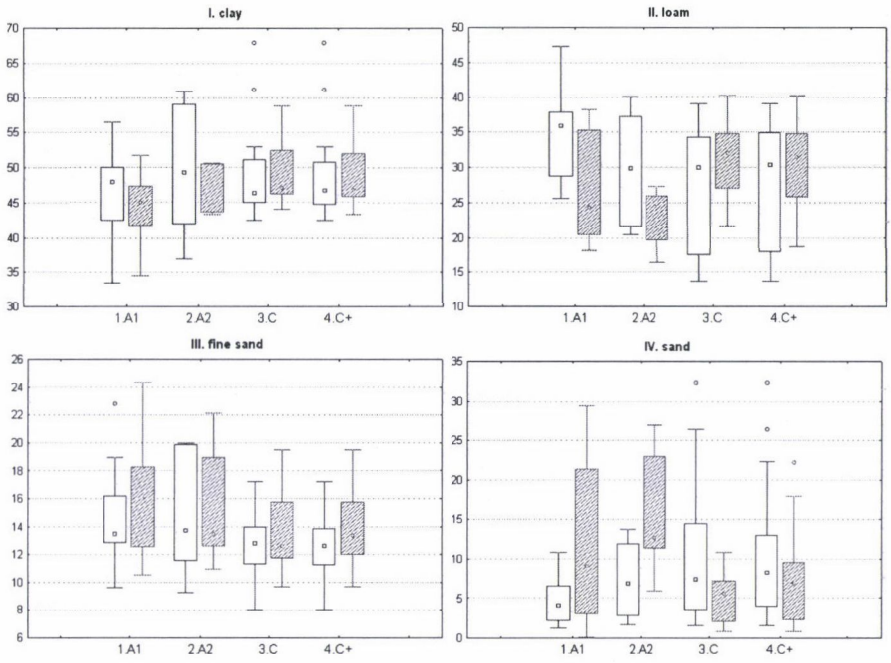



*Fig. 4.* Leptosols, changes in texture, four classes; X-axes show four horizons. For graph design see Fig. 2. The most striking change is in the topsoil horizons (1.A1, 2.A2), where the finer fractions (I and II) have been lost, while the coarse fraction IV increased

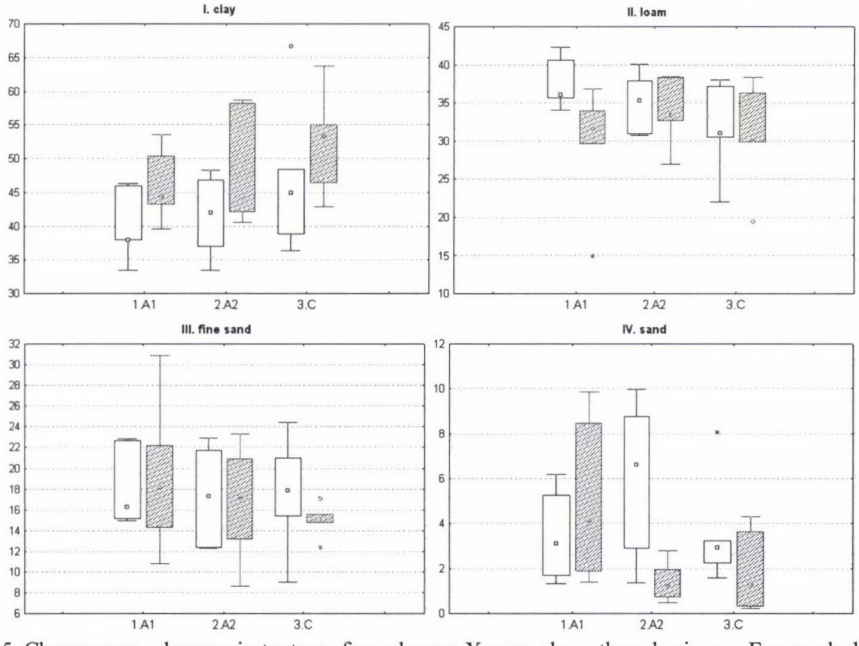



*Fig. 5.* Chernozems, changes in texture, four classes; X-axes show three horizons. For graph design see Fig. 2. The most apparent changes are in fraction I (increase) and IV (both increase and decrease)

*Content of carbonates*

Low or even zero absolute values were recorded in most horizons, except for the substrate (C) horizons, but the changes were often statistically significant, including the statistically significant ($p < 0.05$) decrease in the C-horizon of Luvisols (Fig. 6) and the upper horizons of Regosols (Fig. 7), and the increase in the uppermost horizon of Leptosols (Fig. 8).

*Organic matter content*

No statistically significant changes. A slight increase could be observed in the topsoil horizons of Leptosols (Fig. 8) and Luvisols (Fig. 6), but there was no change in subsoil horizons containing a low amount of organic matter.

*pH in $H_2O$*

The overall change was an increase in almost all horizons, most notably in those of Leptosols (Fig. 8), which could be as much as 0.4 units. There was a statistically highly significant ($p < 0.01$) increase in Leptosols in most horizons, and a significant ($p < 0.05$) increase in Regosols, except for the uppermost horizon. In contrast, Luvisols (Fig. 6) changed the least, with practically no change in the topsoil horizons. A slight increase in acidity was observed in Chernozems.

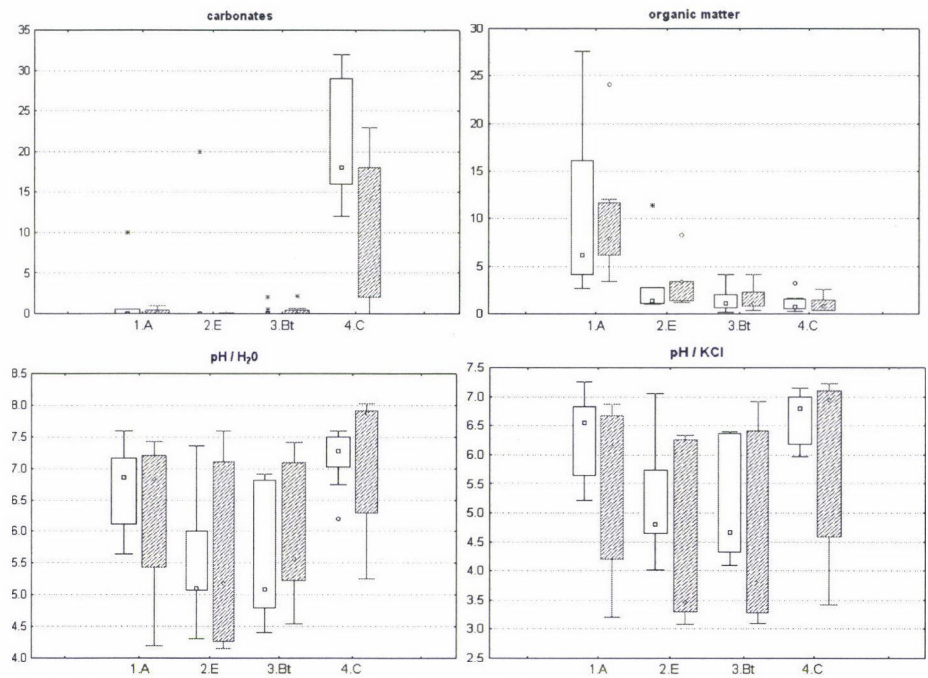



*Fig. 6.* Luvisols, changes in content of carbonates (%), content of organic matter (%), pH in $H_2O$ and pH in 1 N KCl solution. X-axes show four horizons; for graph design see Fig. 2. The most interesting process is the decrease in pH/KCl in luvic horizons (2.E, 3.Bt) compared to almost no change for pH/$H_2O$

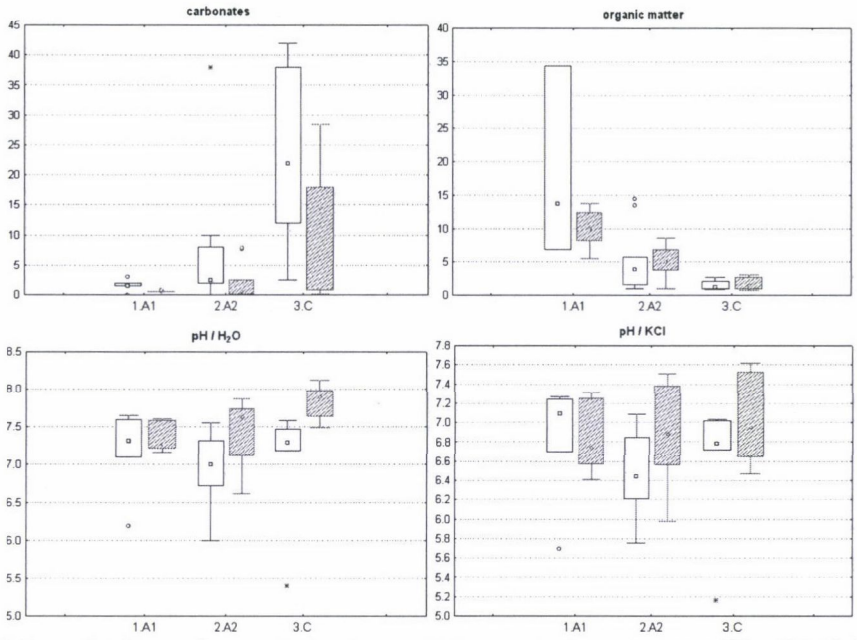



*Fig. 7.* Regosols, changes in content of carbonates (%), content of organic matter (%), pH in $H_2O$ and pH in 1 N KCl solution. X-axes show three horizons; for graph design see Fig. 2. General loss of carbonates (mainly in topsoil) and increase in acidity (mainly in subsoil) are the most apparent changes

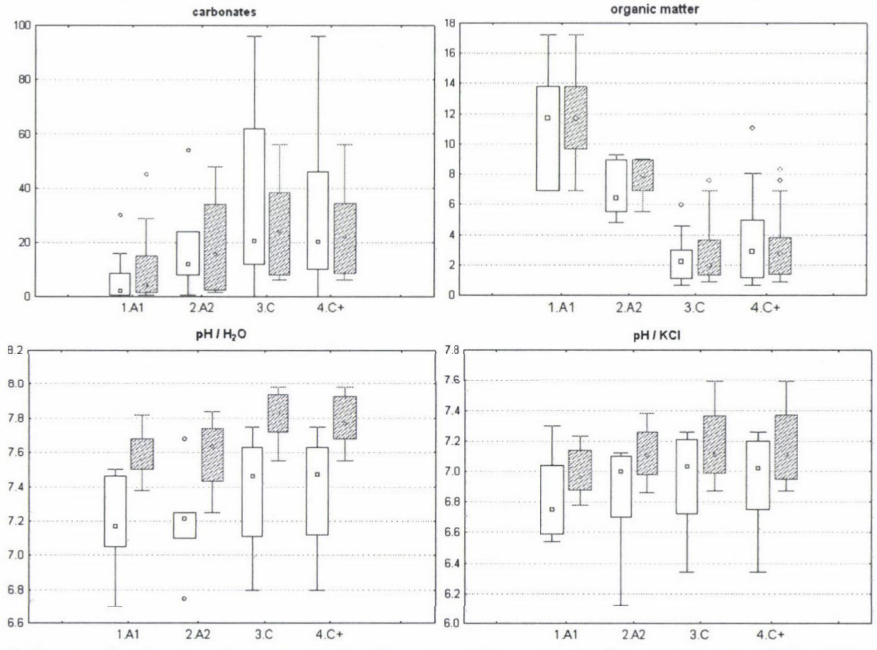



*Fig. 8.* Leptosols, changes in content of carbonates (%), content of organic matter (%), pH in $H_2O$ and pH in 1 N KCl solution. X-axes show four horizons; for graph design see Fig. 2. The most profound change is the increase in acidity in all horizons

*pH in KCl*

No general pattern such as that observed for acidity in $H_2O$ was recorded. An increase of 0.1 to 0.2 units was observed in Leptosols (Fig. 8) and Regosols (Fig. 7), except for the upper horizon. There was a statistically significant ($p < 0.05$) increase in the subsoil of Leptosols. The decrease was not statistically significant in Luvisols (Fig. 6), where it was as much as 1.0 unit in the eluvial (E) horizons.

## Discussion

*Dynamics of soil properties and the woodland succession*

The methodology of the field and laboratory investigations performed in the early 60s was strictly followed in order to eliminate the impacts of the high spatial heterogeneity of the soils and the incompatibility of different laboratory methods. Soil development is of both a continual and discontinual nature. The discontinual processes can be viewed as disturbances in the continual development of the soil and include local episodical catastrophes such as short-term massive erosion events or landslides. Such events (Boero et al., 1992) are independent of the presence of forests, though forests can diminish the effects of erosive acts.

When interpreting the results, consideration was given to the higher consumption of available nutrients due to the higher volume of biomass (Van Breemen, 1993; Ae et al., 2001; Puhe and Ulrich, 2001), chemical alterations in the topsoil (Cresser et al., 1993; Violante et al., 2002), the impacts of changed local hydrology due to lower evaporation and higher transpiration and canopy interception (Dolman et al., 2001) and the altered cycling of nutrients in terms of quicker decomposition of dead plant organic matter (Van der Putten, 1997; Mengel and Kirkby, 2001). Based on the current approach to the dynamics of soil processes (Bonneau and Souchier, 1982; Foth, 1991; Brady and Weil, 1999; Sumner, 2000), it was assumed that the topsoil did not suffer significant drying out under the experimental conditions, thus allowing intense decomposition and the higher production of organic acids, which then infiltrate into the surface/subsurface horizons. Simultaneously, local natural acidification may occur (Bormann and Likens, 1979; Meiwes et al., 1986; Binkley and Richter, 1987; Bredemeier et al., 1990; Lükewille et al., 1993; Puhe and Ulrich, 2001), which is, however, partly/completely buffered by carbonates.

In addition, these processes are influenced by the input of atmospheric pollutants rich in nitrogen into the topsoil (Kauppi et al., 1990; Hofmeister et al., 2002; Hruška et al., 2002; 2003). Differences in the distribution of precipitation throughout the year can be expected as a result of global climate changes (Kalvová, 2000; Puhe and Ulrich, 2001; Rejšek, 2004), possibly leading to local landslides.

*Interpretation of observed changes*

Each of the ten hypotheses was tested against the results of the measurements. Most hypotheses had to be rejected. Most soil units remained unchanged, or at least the change was not statistically significant. For the formulation of the hypotheses, see Introduction.

i) $H_1$: rejected. Vertical transport of clay in the luvic horizons of Luvisols was not detected.

The data confirmed that textural changes are among the slowest soil processes. Within the process of illimerization, the vertical migration of the finest particles from the topsoil to the subsoil should result in an increase in clay in the neighbouring subsoil horizon. Retallack (2001) described how luvic and argilic horizons evolve over hundreds and thousands of years. Holliday (1988) stated that an argillic horizon may evolve within 450 years. The results proved that luvic processes cannot be documented with the data presented.

ii) $H_2$: rejected. Texture changes were observed in all soil types. When evaluating the interrelationships between natural plant succession and the dynamics of soil properties, it must not be forgotten that physical, chemical and biological soil properties are also acquired in response to external biotic factors (Karlen et al., 2003). The data presented showed that the succession of plant communities towards the closed forest has led to the considerably increased erosion of soil profiles and the markedly increased denudation of the Jurassic limestone cliffs; however, the forty-year period was not long enough to produce changes distinguishable at the pedon level.

iii) $H_3$: rejected. Texture changes were often detected in other than the clay fraction. The situation seems to be different in the topsoil horizons, which are the subject of processes of (sub)recent erosion and/or sedimentation (Schaetzl and Anderson, 2005). It is necessary to view the textural changes between the four classes (clay to sand) as the elements of a single system, where an increase or decrease in one element (e.g. textural class) results in an opposite change in other elements, within and between the adjacent horizons. In general, textural changes can have a threefold explanation: i) accumulation (or sedimentation), i.e. an increase in any fraction by horizontal migration, which concerns only the surface horizons in recent pedogenesis; ii) dissolution and weathering, i.e. relative loss of any fraction, but mostly of the coarser particles, leading to an increase in finer particles; iii) relative change, i.e. relative decrease or increase linked with one of the above-mentioned processes.

It is probable that the textural changes observed in topsoil horizons are due to the accumulation of coarser fractions, i.e. of sandy particles, in Leptosols and Regosols and of fine clayey fractions in Luvisols and Chernozems. This conclusion can be supported by the topographic position of the respective soil types, where the former (especially Leptosols) develop on the upper, often steep parts of the slopes, under the rocky cliffs, while the latter can be found on the

lower, moderately inclined parts of the slopes and in the foothills. The sedimentation gradient probably resulted in the observed distribution of fine (<2 mm) soil particles. The share of other fractions within the soil types has relatively declined; however, the loss of coarse fractions could also be due to weathering and dissolving, leading to finer particles.

The situation is rather different in the subsoil horizons, where the dissolution and weathering of coarser (sandy) particles has probably led to the general increase in fine fractions (clay and loam). However, slight quasi-illimerization was observed in the Leptosols, which is in contradiction to the high carbonate content in the whole profile.

iv) $H_4$: supported for Regosols and rejected for Luvisols. A significant decrease in carbonate content was observed in the upper horizons of Regosols, while no change was determined in the topsoil of Luvisols. The data proved that changes in carbonate content may occur within a few decades (Juo and Franzluebbers, 2003). Carbonates are soluble in water, if it is neutral to moderately acidic (Ashman and Puri, 2005). In Regosols, the water regime appeared to have a slightly leaching effect, in contrast to the slightly dessicative regime in the 60s. In contrast no leaching of carbonates down the profile (resulting in a decrease) and no accumulation of calcium carbonate particles by horizontal transport down the slope were measured in Luvisols.

v) $H_5$: ambiguous. In Regosols and Luvisols, all horizons, including the substrate C-horizons, showed a decrease in carbonate content, probably due to leaching down the profile. In addition, a slight enrichment in carbonates due to capillarity uplift, which can be expected only at sunny sites without a closed canopy, was measured in the topsoil of Chernozems. Moreover, the increase in carbonate content in the topsoil of Leptosols could most probably be interpreted as the accumulation of fine grains of calcium carbonate.

vi) $H_6$: rejected. No significant increase in organic matter was observed in the uppermost horizons. The humification rate is probably influenced by the altered soil biotic communities linked to plant exudates and the litter of developing plant communities (Kreitz and Anderson, 1997; Vetter et al., 2004). The joint effect of the development of plant associations providing the soil with different quantities and qualities of dead plant organic matter should also be stressed (Violante et al., 2002). However, the data obtained did not prove either the increasing role of humified soil biota or a higher rate of humic substance formation due to the higher rate of litter accumulation expected on the soil surface.

The changes in organic matter content were difficult to interpret. A slight increase was detected in the organic matter content in the surface topsoil horizons of Luvisols and Leptosols but this change was not significant. Nevertheless, the improved humification could be attributed without doubt to the changed conditions of the ecosystem as the closed woodland developed during the succession.

The results indicate the total organic carbon content added to the humus; hence this value can be treated as the intensity of humification, which concerns only the topsoil (A) horizons. In the subsoil, no humus particles should occur unless transported there by incidental pedoturbation, or as the fine roots of trees, which could not be removed from the samples. The changes in comparable horizons were approximately 5–10% in the A-horizons.

vii) $H_7$: supported. No change in organic matter could be detected in the subsoil. If a change in the organic matter content of the subsoil is measured, pedogenetical translocation can be expected to play a major role. The data obtained proved that no prominent changes in infiltration processes, water redistribution or evaporation from the soil surface (Mengel and Kirkby, 2001; Moffat, 2003), which may be reflected as a higher content of humification products in the subsoil, occurred on the study plots. Incidental pedoturbation or the presence of fine tree roots, which could not be removed from the samples, are the other sources for the transport of organic matter into the subsoil. It is important to evaluate the differences in the absolute values: the changes in comparable horizons amounted to approximately 0.5–1.0% in the C-horizons, i.e. as expected, no change occurred in the subsoil horizons.

viii) $H_8$: rejected. No significant decrease in the soil reaction could be detected in Luvisols or Regosols. In the calcium carbonate-rich soils of Děvín, acidification is only to be expected in non-carbonate or carbonate-poor horizons – as in Luvisols and Regosols. The data showed that the soil reaction in Luvisols and Regosols in the study area exhibited long-term stability, even though the authors are fully aware that soil acidity is a dynamic, highly variable parameter (White, 1997). In neither soil unit were any significant changes in pH values observed due to: i) the release of organic acids by humification processes, which is a typical process of woodland succession (Sumner, 2000), leading to a decrease in soil acidity, mostly in the topsoil; ii) carbonate leaching, which is closely linked with the acidity value, leading to a decrease in soil acidity; iii) the anthropogenic input of acidifiers, mediated mainly by precipitation, leading to a decrease in acidity; or iv) the accumulation of calcium carbonate particles by horizontal migration and sedimentation, leading to an increase in soil acidity. In addition, the role of airborne acidification by sulphur and nitrogen oxides of anthropogenic origin in Central Europe (Almer et al., 1974; Sutcliffe et al., 1982) was not proved either.

In Luvisols, both luvic horizons (E and Bt) were, in general, carbonate-free, while the pH reached the moderately acidic to acidic range (with values as low as 3 for pH in KCl). Because they were the most acidic of all horizons treated in general, they should be very sensitive to the input of $H^+$ ions. Nevertheless, the organic acids produced by improved humification and/or the atmospheric deposition of acidifiers were buffered by the sorption complex. Therefore, no significant decrease in acidity could be observed. The same probably holds true for the upper horizon of Regosols.

ix) $H_9$: supported. For both Luvisols and Regosols, the pH in KCl dropped more than the pH in water. A relatively strong decrease in the soil reaction in Luvisols and Regosols was measured in KCl. Soil acidity was measured in two ways: in a distilled water suspension, and in 1 M KCl. Measuring the acidity in water shows the concentration of $H^+$ ions actually present in the soil solution, while KCl treatment also releases $H^+$ ions bound electrochemically on the surface of the soil colloid particles (Courtney and Trudgill, 1993). The import of acidifying ions from outside can be documented by the decreasing value of pH in KCl, although this acid input does not necessarily affect the pH in water. The decrease in pH in an electrolyte (1 M KCl) was no doubt influenced by the local effects of global climate changes (Rejšek, 2004): in this part of Central Europe, the role of a long-term high air pollution rate (Purdon et al., 2004) must be considered. The data presented may also be influenced by continuous variations in soil buffering capacities followed by short-term enhancements in soil acidity (Boyle and Powers, 2001). The authors see the enhancement of exchangeable acidity as a result of both the humification processes and/or precipitation deposits. Based on the data evaluated, it was impossible to distinguish between acidification due to natural succession and acidic pollution.

x) $H_{10}$: rejected. A highly significant increase in the soil reaction was measured in Leptosols. This increase in pH throughout the whole profile in Leptosols was the most striking of all the changes in pH values determined in the study. This dramatic enhancement could be interpreted as the accumulation of calcium carbonate particles in the topsoil and the seeping of the carbonate-rich solution into the subsoil. In KCl, the increase in pH was not as high as in water, since a certain quantity of $H^+$ ions is present in the sorption complex. However, accumulation in the topsoil may be closely linked with other parameters (carbonate content, texture), while the seepage of the alkaline solution is not easy to prove. A slight increase in pH values in Chernozems can most probably be interpreted as the capillarity uplift of the calcium carbonate solution, but this increase was not statistically significant.

## Conclusions

Based on the assumptions and on the soil changes detected, it was concluded that there were at least two Quaternary-geological and three pedogenetic processes:

(1) Gravitative accumulation of limestone particles arising from the continual mechanical denudation of the cliffs, though the transport of particles larger than 2 mm could not be detected by the present data.

(2) Weathering of coarser particles into finer particles in soils located on flat, under-hill sites. Such soils were the only soils displaying mild acidification. Weathering processes could be documented both in the parent materials and inside soil horizons.

(3) Changes in humidity, temperature and wind speed on the soil surface had a great influence on the dynamics of soil properties by dramatically reducing soil drying-up periods. This could only be detected indirectly.

(4) An improvement in the humification rate, resulting in mild acidification in non-calcareous horizons. This acidification cannot be distinguished from the effect of atmospheric depositions, mainly nitrogen compounds, which are certainly present.

(5) Although gravitative clay migration probably takes place, the 40-year period was too short to offer evidence for clayey particle translocations between adjacent horizons.

## Acknowledgements

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*Appendix*

Soil profiles, sampled horizons, soil types and results of laboratory analyses for texture (content of four classes, %), content of carbonates (%), content of organic matter (%), and acidity in distilled water and 1 N KCl solution. Pairs of related values are given; Old denotes values taken from the literature, New denotes values measured in recently taken samples. Blank fields are due to missing values (not measured)

| Plot No. | Depth (cm) | Soil type | Horizon | Texture classes I | | II | | III | | IV | | Carbonates | | Organic matter | | pH in $H_2O$ | | pH in KCl | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | old | new | old | new | old | new | old | new | old | new | old | new | old | new | old | new |
| Luvisols | | | | | | | | | | | | | | | | | | | |
| 406 | 1–10 | HL | Ah | 41.1 | 50.2 | 38.7 | 34.5 | 17.6 | 14.1 | 2.7 | 1.3 | 0.5 | 1 | 6.90 | 8.28 | 6.70 | 7.21 | 6.40 | 6.87 |
| | 10–15 | | AE | 46.1 | 57.9 | 36.8 | 28.9 | 14.1 | 11.6 | 3.0 | 1.6 | 0 | 0 | 1.15 | 3.45 | 5.07 | 4.72 | 4.81 | 3.47 |
| | 25–30 | | E | 46.7 | 56.1 | 35.3 | 32.1 | 15.4 | 11.5 | 2.7 | 0.2 | 0 | 0 | 1.03 | 1.17 | 4.31 | 4.16 | 4.02 | 3.36 |
| | 40–50 | | Bt | 55.8 | 57.3 | 30.9 | 28.2 | 11.5 | 11.0 | 1.8 | 3.4 | 0 | 2.2 | 0.41 | 0.48 | 4.41 | 7.10 | 4.10 | 6.92 |
| | 70–80 | | Ck | 55.4 | 57.8 | 27.1 | 30.9 | 12.0 | 8.7 | 5.5 | 2.5 | 12 | 23 | 0.34 | 0.34 | 6.20 | 7.89 | 5.97 | 7.22 |
| 421 | 0–3 | HL | Ah | 45.7 | 41.3 | 35.1 | 39.8 | 14.4 | 18.0 | 4.7 | 0.8 | 10 | 0.05 | 16.1 | 11.73 | 7.50 | 7.43 | 7.25 | 6.35 |
| | 15–20 | | AE | 43.5 | 50.9 | 34.0 | 44.7 | 13.7 | 4.3 | 8.8 | 0.2 | 20 | 0.02 | 11.37 | 2.76 | 7.36 | 7.59 | 7.05 | 6.33 |
| | 80–90 | | Ck | 47.0 | 40.0 | 33.2 | 50.2 | 13.1 | 9.1 | 6.6 | 0.7 | 32 | 2.1 | 1.65 | 1.59 | 7.59 | 7.87 | 7.15 | 6.80 |
| 422 | 5–10 | HL | Ah | 40.2 | 45.7 | 40.0 | 44.2 | 17.2 | 9.0 | 2.7 | 1.1 | 0 | 0.1 | 5.50 | 6.21 | 7.02 | 7.20 | 6.82 | 6.40 |
| | 20–30 | | AB | 42.0 | 38.0 | 42.1 | 38.5 | 14.1 | 21.3 | 1.7 | 2.2 | 0 | 0 | 1.38 | 3.45 | 6.00 | 7.11 | 5.73 | 6.26 |
| | 45–55 | | Bt | 46.8 | 42.8 | 37.9 | 45.3 | 14.3 | 11.0 | 1.0 | 0.8 | 0 | 0.1 | 0.69 | 1.10 | 6.15 | 7.28 | 5.70 | 6.17 |
| | 90–100 | | Ck | 49.6 | 40.7 | 34.9 | 42.6 | 12.8 | 16.5 | 2.7 | 0.2 | 20 | 6.8 | 0.57 | 0.69 | 7.50 | 7.91 | 7.00 | 6.99 |
| 423 | 30–40 | HL | Bt | | | | | | | | | | | | 2.07 | 4.14 | 6.43 | 6.17 | 6.03 | 5.10 |
| | 50–60 | | Ck | | | | | | | | | | | | 1.26 | 1.52 | 6.74 | 6.30 | 6.12 | 4.59 |
| 424 | 2–5 | HL | Ah | 69.5 | 49.5 | 24.4 | 29.3 | 5.5 | 18.3 | 0.6 | 2.9 | 0.5 | 0.05 | 13.8 | 12.08 | 7.17 | 6.28 | 6.71 | 5.87 |
| | 30–40 | | Bt | 76.6 | 51.4 | 17.7 | 32.8 | 5.1 | 14.8 | 0.5 | 1.0 | 2 | 0.03 | 4.13 | 3.68 | 6.82 | 5.56 | 6.36 | 3.74 |
| | 55–60 | | C | 66.0 | 60.2 | 20.8 | 31.7 | 11.0 | 7.3 | 2.2 | 0.7 | 18 | 0 | 0.70 | 2.62 | 7.30 | 5.26 | 6.94 | 3.42 |

| | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 426 | 1–2 | HL | O+Ah | | | | | | | | 0 | 0 | 27.6 | 24.15 | 7.60 | 5.16 | 7.04 | 4.11 |
| | 2–3 | | Ah | 35.9 | 47.3 | 35.2 | 42.0 | 27.0 | 9.5 | 1.9 | 1.1 | 0 | 0 | 20.7 | 11.04 | 5.92 | 4.20 | 5.50 | 3.20 |
| | 5–15 | | E | 43.0 | 43.9 | 40.0 | 44.1 | 14.5 | 10.8 | 2.5 | 1.3 | 0 | 0 | 2.76 | 8.28 | 5.10 | 4.26 | 4.70 | 3.09 |
| | 25–30 | | Bt1 | 53.1 | 57.2 | 31.2 | 42.5 | 13.3 | 0.0 | 2.4 | 0.2 | 0 | 0 | 1.15 | 2.30 | 4.64 | 4.54 | 4.30 | 3.10 |
| | 50–60 | | Bt2 | 57.8 | 46.0 | 30.6 | 40.1 | 10.2 | 12.7 | 1.4 | 1.2 | 0 | 0 | 0.17 | 0.83 | 4.90 | 5.23 | 4.57 | 3.32 |
| 427 | 5–10 | HL | Ah | 37.1 | 41.7 | 44.5 | 40.5 | 17.3 | 16.6 | 1.1 | 1.3 | 0 | 0.1 | 2.70 | 7.59 | 5.65 | 6.65 | 5.22 | 5.96 |
| | 20–30 | | AB | 41.2 | 37.3 | 40.0 | 24.7 | 17.3 | 21.3 | 1.5 | 16.7 | 0 | 0.1 | 1.38 | 3.45 | 5.07 | 6.04 | 4.65 | 5.09 |
| | 55–65 | | Bt | 45.7 | 44.2 | 34.3 | 28.4 | 18.6 | 17.7 | 1.4 | 9.8 | 0 | 0 | 0.69 | 0.97 | 5.02 | 5.54 | 4.67 | 3.84 |
| | 90–100 | | Ck | 44.8 | 50.7 | 36.6 | 46.5 | 17.4 | 2.4 | 1.2 | 0.4 | 32 | 18 | 0.27 | 0.69 | 7.20 | 7.87 | 6.18 | 6.97 |
| 429 | 3–6 | HL | Ah | 35.1 | 39.6 | 43.3 | 33.8 | 20.1 | 15.3 | 1.4 | 11.3 | 0 | 0 | 5.50 | 4.14 | 6.12 | 5.44 | 5.64 | 4.21 |
| | 10–15 | | E | 35.6 | 41.8 | 46.7 | 30.5 | 17.0 | 20.3 | 0.7 | 7.3 | 0 | 0.05 | 2.07 | 1.38 | 5.17 | 5.19 | 4.80 | 3.30 |
| | 30–40 | | Bt1 | 41.0 | 47.1 | 41.7 | 28.7 | 16.5 | 16.7 | 0.7 | 7.5 | 0 | 0 | 1.72 | 1.03 | 4.80 | 4.98 | 4.33 | 3.28 |
| | 50–60 | | Bt2 | 42.2 | 40.9 | 38.9 | 36.1 | 17.9 | 15.9 | 1.0 | 7.1 | 0 | 0 | 1.15 | 0.41 | 5.09 | 5.24 | 4.67 | 3.27 |
| | 80–90 | | BC | 35.3 | 46.7 | 41.0 | 39.7 | 21.8 | 10.6 | 1.9 | 3.0 | 14 | 0 | 0.55 | 0.34 | 7.02 | 5.71 | 6.19 | 3.64 |
| 436 | 3–8 | HL | Ah | 43.4 | 44.3 | 37.0 | 37.6 | 17.8 | 14.6 | 1.8 | 3.5 | 0.5 | 0.65 | 2.70 | 3.45 | 6.47 | 7.00 | 6.02 | 6.72 |
| | 20–30 | | Bt | 47.8 | 45.6 | 36.9 | 35.5 | 13.4 | 18.4 | 1.9 | 0.6 | 0.5 | 0.4 | 2.07 | 2.07 | 6.90 | 7.42 | 6.37 | 6.78 |
| | 50–70 | | C1 | 42.8 | 47.1 | 33.8 | 35.9 | 17.4 | 13.6 | 6.0 | 3.4 | 16 | 14 | 1.61 | 1.03 | 7.26 | 7.95 | 6.76 | 7.10 |
| | 80–90 | | C2 | 41.4 | 48.6 | 33.6 | 37.0 | 16.6 | 11.9 | 8.3 | 2.5 | 29 | 20.5 | 0.69 | 0.34 | 7.40 | 8.03 | 6.83 | 7.21 |
| 437 | 2–10 | HL | Ah | 42.1 | 47.0 | 37.1 | 30.1 | 17.5 | 11.0 | 3.3 | 11.9 | 0 | 0.4 | 4.10 | 6.90 | 7.10 | 7.04 | 6.80 | 6.67 |
| | 20–30 | | Bt | 41.5 | 47.9 | 39.8 | 37.0 | 15.4 | 14.1 | 3.3 | 1.0 | 0 | 0.6 | 1.38 | 1.73 | 6.91 | 7.07 | 6.40 | 6.41 |
| | 60–70 | | Ck | 44.3 | 48.5 | 34.1 | 31.3 | 15.2 | 12.3 | 6.3 | 7.8 | 18 | 15.5 | 3.21 | 1.21 | 7.52 | 7.81 | 7.10 | 6.92 |
| Regosols | | | | | | | | | | | | | | | | | | | |
| 415 | 0–10 | CR | Ah1 | 45.4 | 40.9 | 40.4 | 23.5 | 11.8 | 14.7 | 2.4 | 20.9 | 0 | 0.5 | 6.90 | 10.35 | 6.19 | 7.59 | 5.70 | 7.31 |
| | 20–30 | | Ah2 | 48.0 | 45.7 | 35.1 | 40.3 | 11.3 | 13.3 | 5.6 | 0.8 | 5 | 2 | 3.44 | 4.14 | 6.40 | 7.61 | 6.07 | 7.51 |
| | 60–70 | | Ah3 | 49.3 | 47.6 | 39.9 | 41.7 | 10.3 | 9.3 | 0.6 | 1.5 | 2 | 2.5 | 1.03 | 3.79 | 6.00 | 7.74 | 5.76 | 7.50 |
| | 85–100 | | Ck1 | 43.6 | 48.5 | 42.0 | 39.3 | 13.5 | 11.1 | 0.8 | 1.1 | 2.5 | 4 | 0.86 | 1.72 | 5.40 | 7.83 | 5.16 | 7.53 |
| 431 | 0–3 | CR | Ah1 | 45.2 | 36.0 | 34.8 | 18.1 | 16.4 | 17.0 | 3.5 | 28.9 | 3 | 0.8 | 13.8 | 13.8 | 7.51 | 7.17 | 7.27 | 6.85 |
| | 20–30 | | Ah2 | 45.3 | 39.4 | 37.8 | 27.9 | 14.5 | 19.0 | 2.5 | 13.7 | 8 | 7.8 | 5.74 | 8.62 | 7.30 | 7.65 | 6.74 | 7.38 |
| 432 | 0–3 | CR | Ah1 | 44.3 | 49.1 | 34.8 | 36.0 | 17.5 | 13.9 | 3.4 | 1.1 | 1.5 | 0.5 | 6.90 | 5.52 | 7.60 | 7.28 | 7.10 | 6.41 |
| | 10–25 | | Ah2 | 41.4 | 46.2 | 36.6 | 34.8 | 18.8 | 16.6 | 3.2 | 2.3 | 3 | 0.3 | 4.48 | 2.42 | 7.32 | 7.34 | 6.85 | 6.56 |
| | 50–60 | | Ck | 50.8 | 46.6 | 25.4 | 38.6 | 19.7 | 13.3 | 4.2 | 1.5 | 38 | 0.2 | 1.18 | 1.52 | 7.19 | 7.49 | 6.72 | 6.65 |
| 441 | 0–5 | CR | Ah1 | 54.9 | 43.4 | 31.3 | 41.5 | 12.0 | 13.9 | 1.8 | 1.2 | 2 | 0.5 | 13.8 | 8.28 | 7.65 | 7.22 | 7.24 | 6.74 |
| | 15–25 | | Ah2 | 55.8 | 54.5 | 29.6 | 39.1 | 12.2 | 5.0 | 2.4 | 1.4 | 10 | 0.1 | 13.45 | 7.59 | 7.47 | 6.61 | 7.00 | 5.98 |
| | 50–60 | | Ah3 | 71.7 | 46.0 | 18.4 | 39.4 | 9.1 | 13.4 | 0.8 | 1.1 | 38 | 7.9 | 0.96 | 1.03 | 7.56 | 7.87 | 7.09 | 6.93 |
| 442 | 5–10 | CR | Ah1 | | | | | | | | | | | | | | 7.20 | 7.22 | 6.70 | 6.57 |
| | 10–20 | | Ah2 | 49.3 | 46.3 | 36.5 | 34.4 | 12.5 | 18.9 | 1.7 | 0.4 | 0 | 0.5 | 2.76 | 5.86 | 6.83 | 7.11 | 6.24 | 6.63 |
| | 20–30 | | Ah3 | 51.3 | 45.8 | 34.0 | 36.3 | 12.6 | 16.2 | 2.1 | 1.7 | 0 | 0.1 | 1.61 | 3.80 | 6.74 | 7.12 | 6.29 | 6.23 |
| | 40–50 | | Ck1 | 49.5 | 48.1 | 34.1 | 38.6 | 11.0 | 13.1 | 5.4 | 0.2 | 12 | 0.8 | 1.51 | 2.76 | 7.42 | 7.64 | 6.78 | 6.47 |
| | 70–80 | | Ck2 | 45.8 | 48.1 | 34.1 | 37.2 | 15.0 | 10.4 | 5.1 | 4.3 | 42 | 18 | 0.98 | 1.38 | 7.59 | 7.95 | 7.04 | 6.95 |
| 443 | 0–5 | CR | Ah1 | 39.2 | 45.8 | 47.3 | 36.6 | 12.1 | 16.7 | 1.4 | 0.8 | 1.5 | 0.6 | 34.4 | 12.42 | 7.32 | 7.42 | 7.22 | 6.70 |
| | 10–20 | | Ah2 | 59.5 | 48.7 | 29.2 | 40.7 | 10.3 | 9.8 | 1.0 | 0.8 | 2 | 0.6 | 14.47 | 6.90 | 7.17 | 7.74 | 6.60 | 6.84 |
| | 50–60 | | Ck | 60.8 | 45.8 | 22.2 | 42.3 | 13.5 | 11.8 | 3.5 | 0.1 | 29 | 14.5 | 2.07 | 3.10 | 7.30 | 8.12 | 6.79 | 6.95 |
| 129a | 0–10 | CR | Ah1 | 48.2 | 43.7 | 34.0 | 24.9 | 14.9 | 16.7 | 3.0 | 14.7 | 1.5 | 0.5 | 34.4 | 9.66 | 7.10 | 7.61 | 6.96 | 7.25 |
| | 25–35 | | Ah2 | 48.3 | 44.6 | 31.4 | 34.4 | 16.9 | 18.1 | 3.5 | 2.9 | 2 | 0.2 | 5.17 | 5.86 | 6.72 | 7.65 | 6.21 | 7.34 |
| | 60–70 | | Ck1 | 42.7 | 47.2 | 31.0 | 35.3 | 20.4 | 15.6 | 5.9 | 2.0 | 19 | 17 | 2.66 | 0.97 | 7.25 | 7.89 | 6.86 | 7.51 |
| | 90–100 | | Ck2 | 39.5 | 42.9 | 30.5 | 32.4 | 23.9 | 18.9 | 6.1 | 5.7 | 22 | 28.5 | 1.11 | 0.76 | 7.47 | 7.98 | 7.02 | 7.62 |
| Leptosols | | | | | | | | | | | | | | | | | | | |
| 404 | 2–10 | ML | Ah1 | 51.4 | 45.3 | 25.6 | 23.9 | 14.1 | 24.3 | 8.9 | 6.4 | 16 | 29 | 6.90 | 8.97 | 7.15 | 7.82 | 6.75 | 7.23 |
| | 20–25 | | Ah2 | 52.8 | 46.1 | 21.6 | 25.9 | 13.7 | 22.1 | 11.9 | 5.9 | 24 | 34 | 4.82 | 5.52 | 7.24 | 7.72 | 7.00 | 7.38 |
| | 60–70 | | C | 67.9 | 46.2 | 17.6 | 27.0 | 12.8 | 19.5 | 1.7 | 7.2 | 23 | 47 | 0.96 | 0.90 | 7.40 | 7.68 | 7.02 | 7.51 |
| 408 | 2–5 | EL | Ah1 | 33.5 | 47.9 | 42.4 | 30.0 | 22.8 | 18.8 | 1.3 | 3.3 | 7.5 | 20 | 10.3 | | | | | |
| | 10–15 | | Ah2 | 37.0 | 44.3 | 40.1 | 27.4 | 20.0 | 15.6 | 2.9 | 12.7 | 12 | 25 | 6.90 | | | | | |
| | 40–50 | | Ck | 45.0 | 47.9 | 38.0 | 34.8 | 15.4 | 15.2 | 1.6 | 2.2 | 20.5 | 24 | 2.76 | | | | | |
| 411 | 0–3 | ML | Ah1 | 48.1 | 44.2 | 29.1 | 18.1 | 17.6 | 12.6 | 5.1 | 25.0 | 2.5 | 3.5 | 6.90 | 13.8 | 6.92 | 7.64 | 6.90 | 7.20 |
| | 20–30 | | Ah2 | 49.2 | 44.8 | 29.9 | 19.7 | 14.0 | 12.6 | 6.8 | 22.9 | 8 | 9 | 5.51 | 7.59 | 7.10 | 7.55 | 7.00 | 7.26 |
| | 60–70 | | Ck | 46.4 | 53.4 | 27.8 | 31.2 | 11.4 | 11.1 | 14.5 | 4.2 | 12 | 38.5 | 2.89 | 3.45 | 7.39 | 7.82 | 7.04 | 7.59 |
| 412 | 0–5 | ML | Ah1 | 48.7 | 44.6 | 35.6 | 37.6 | 13.3 | 17.7 | 2.5 | 0.1 | 1 | 0.5 | 16.1 | 17.25 | 6.70 | 7.56 | 6.68 | 7.04 |
| | 25–35 | | Ah2 | 48.2 | 50.5 | 37.3 | 24.7 | 11.6 | 13.0 | 2.9 | 11.8 | 10 | 2.4 | 8.96 | 9.00 | 7.25 | 7.25 | 7.10 | 6.98 |
| | 60–70 | | Ck | 50.5 | 46.5 | 30.0 | 29.7 | 8.0 | 14.9 | 11.5 | 8.9 | 30 | 15 | 2.89 | 3.79 | 7.63 | 7.76 | 7.22 | 7.34 |

| | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 414 | 0–3 | ML | Ah | 44.0 | 34.5 | 38.2 | 18.8 | 13.3 | 17.3 | 4.5 | 29.4 | 2 | 5 | 17.2 | 10.35 | 7.17 | 7.70 | 7.03 | 7.06 |
| | 30–40 | | AC | 44.5 | 43.2 | 38.9 | 25.4 | 12.0 | 13.4 | 4.5 | 18.0 | 8 | 10.5 | 4.99 | 3.45 | 7.18 | 7.77 | 6.86 | 7.37 |
| 435 | 5–10 | EL | Ah | 35.8 | 43.6 | 47.3 | 38.2 | 14.8 | 15.2 | 2.2 | 3.1 | 0 | 2 | 9.65 | 11.73 | 7.09 | 7.38 | 6.70 | 6.83 |
| | 25–30 | | Bw | 51.1 | 52.5 | 35.6 | 36.9 | 11.2 | 9.7 | 2.0 | 0.9 | 0 | 7.8 | 3.10 | 6.90 | 6.80 | 7.55 | 6.34 | 6.93 |
| | 50–60 | | Ck | 46.2 | 46.0 | 30.8 | 34.8 | 14.0 | 12.6 | 9.1 | 6.7 | 18 | 20.5 | 0.69 | 1.90 | 7.12 | 7.84 | 6.70 | 7.11 |
| 446 | 0–3 | RL | Ah1 | 56.5 | 38.0 | 30.2 | 18.7 | 9.6 | 14.3 | 3.6 | 29.1 | 6 | 10 | 13.8 | 13.8 | 7.22 | 7.55 | 6.58 | 6.89 |
| | 15–20 | | Ah2 | 60.9 | 43.2 | 20.4 | 16.3 | 11.8 | 13.5 | 6.8 | 26.9 | 18 | 15.5 | 9.30 | 6.90 | 7.19 | 7.74 | 6.70 | 6.98 |
| | 20–40 | | AC | 49.2 | 45.7 | 26.5 | 18.7 | 13.1 | 13.4 | 11.3 | 22.2 | 26 | 30 | 8.04 | 3.10 | 7.57 | 7.72 | 6.99 | 7.04 |
| | 60–70 | | Ck | 44.3 | 53.4 | 16.1 | 23.9 | 17.2 | 12.6 | 22.3 | 10.1 | 62 | 39 | 1.03 | 1.10 | 7.52 | 7.96 | 7.02 | 7.10 |
| 447 | 0–3 | RL | Ah1 | 42.7 | 39.9 | 27.5 | 24.7 | 19.0 | 23.6 | 10.8 | 11.9 | 30 | 45 | 13.09 | 11.73 | 7.50 | 7.68 | 7.04 | 7.14 |
| | 20–30 | | Ah2 | 41.9 | 43.7 | 24.4 | 26.0 | 19.9 | 18.9 | 13.8 | 11.4 | 54 | 48 | 6.66 | 8.28 | 7.68 | 7.84 | 7.12 | 7.23 |
| | 70–80 | | Ck | 42.4 | 45.9 | 14.2 | 26.2 | 17.1 | 17.0 | 26.4 | 10.9 | 76 | 56 | 1.28 | 1.79 | 7.75 | 7.93 | 7.22 | 7.39 |
| 451 | 5–10 | EL | Ah | 47.6 | 45.4 | 37.6 | 33.1 | 11.7 | 16.9 | 3.1 | 4.6 | 2 | 9.5 | 11.71 | 12.42 | 7.46 | 7.67 | 7.13 | 6.96 |
| | 30–40 | | Bw | 44.4 | 51.5 | 39.0 | 36.6 | 11.3 | 10.2 | 5.3 | 1.7 | 7 | 7 | 5.97 | 7.59 | 7.63 | 7.91 | 7.26 | 7.05 |
| | 70–80 | | Ck | 47.1 | 47.0 | 34.3 | 40.2 | 13.7 | 11.8 | 4.9 | 1.0 | 20 | 8 | 2.56 | 2.76 | 7.60 | 7.95 | 7.20 | 7.11 |
| 452 | 0–5 | RL | Ah | 42.0 | 51.7 | 36.3 | 37.5 | 13.7 | 10.6 | 8.0 | 0.2 | 10 | 3.5 | 13.80 | 17.25 | 7.48 | 7.50 | 7.30 | 6.90 |
| | 15–25 | | AC | 48.0 | 45.4 | 31.1 | 31.7 | 11.1 | 15.8 | 9.8 | 7.1 | 13 | 9 | 11.02 | 8.28 | 7.47 | 7.61 | 7.07 | 6.95 |
| 453 | 5–10 | EL | Ah | 48.1 | 46.6 | 37.3 | 23.5 | 12.5 | 12.1 | 2.1 | 17.8 | 0.5 | 0.75 | 6.89 | 6.90 | 7.05 | 7.50 | 6.54 | 6.78 |
| | 15–25 | | AB | 53.0 | 47.1 | 31.0 | 34.5 | 12.4 | 15.8 | 3.5 | 2.6 | 2 | 6.2 | 4.59 | 2.07 | 7.00 | 7.76 | 6.49 | 6.87 |
| | 60–80 | | Ck | 61.2 | 44.0 | 18.4 | 32.1 | 13.0 | 18.3 | 7.4 | 5.6 | 92 | 26 | 1.91 | 1.24 | 7.66 | 7.98 | 7.11 | 7.24 |
| 455 | 5–10 | ML | Ah1 | 53.5 | 51.3 | 28.4 | 22.0 | 13.3 | 12.6 | 4.9 | 14.1 | 0.5 | 1.3 | 11.71 | 9.66 | 7.22 | 7.42 | 6.59 | 6.88 |
| | 15–25 | | Ah2 | 59.1 | 50.7 | 29.9 | 21.8 | 9.3 | 11.0 | 1.8 | 16.5 | 0.5 | 1.5 | 6.20 | 8.97 | 6.75 | 7.43 | 6.12 | 6.86 |
| | 50–60 | | Ck | 45.3 | 58.8 | 13.7 | 21.7 | 8.7 | 12.3 | 32.3 | 7.2 | 96 | 26 | 1.17 | 1.37 | 7.10 | 7.65 | 6.75 | 6.90 |
| Chernozems | | | | | | | | | | | | | | | | | | | |
| 405 | 1–10 | HC | Ah | 38.0 | 53.5 | 34.0 | 31.6 | 22.7 | 10.8 | 5.3 | 4.1 | 1.5 | 5 | 8.30 | 6.90 | 7.19 | 7.63 | 6.50 | 7.31 |
| | 25–30 | | AC | 38.1 | 58.7 | 31.0 | 27.0 | 22.9 | 13.1 | 8.0 | 1.2 | 2 | 9.5 | 3.79 | 2.76 | 7.12 | 7.67 | 6.74 | 7.43 |
| | 60–75 | | Ck | 48.4 | 55.0 | 30.5 | 29.8 | 17.9 | 15.0 | 3.2 | 0.2 | 8 | 31 | 1.24 | 0.83 | 7.60 | 8.40 | 7.19 | 7.67 |
| 407 | 2–5 | HC | Ah1 | 33.5 | 44.3 | 42.4 | 14.9 | 22.8 | 30.9 | 1.3 | 9.9 | 7.5 | 20.5 | 12.4 | | | | | |
| | 10–15 | | Ah2 | 37.0 | 43.6 | 40.1 | 33.6 | 20.0 | 20.9 | 2.9 | 2.0 | 12 | 24.5 | 4.59 | | | | | |
| | 40–50 | | Ck | 45.0 | 63.7 | 38.0 | 19.5 | 15.4 | 15.5 | 1.6 | 1.3 | 20.5 | 30 | 1.08 | | | | | |
| 409 | 2–5 | HC | Ah1 | 45.9 | 50.4 | 36.1 | 29.7 | 16.3 | 18.1 | 1.7 | 1.9 | 1 | 11 | 12.4 | | | | | |
| | 10–15 | | Ah2 | 48.3 | 44.9 | 37.9 | 38.3 | 12.4 | 16.0 | 1.4 | 0.7 | 3.5 | 26 | 5.34 | | | | | |
| | 40–50 | | Ck | 38.9 | 46.5 | 37.2 | 38.4 | 21.0 | 14.8 | 3.0 | 0.3 | 18 | 18 | 1.57 | | | | | |
| 420 | 2–5 | LC | Ah1 | 46.3 | 39.5 | 35.7 | 36.9 | 14.9 | 22.2 | 3.1 | 1.4 | 6 | 5.4 | 6.90 | 8.28 | 7.35 | 7.49 | 7.02 | 7.20 |
| | 20–30 | | Ah2 | 46.8 | 42.1 | 35.7 | 38.5 | 12.3 | 18.1 | 5.2 | 1.3 | 10 | 6.4 | 6.20 | 6.90 | 7.40 | 7.68 | 7.09 | 7.12 |
| | 50–60 | | Bt | 45.9 | 58.2 | 30.7 | 32.7 | 14.6 | 8.7 | 8.8 | 0.5 | 34 | 32 | 2.53 | 1.79 | 7.39 | 8.06 | 7.05 | 7.20 |
| | 80–90 | | Ck | 66.7 | 53.3 | 22.0 | 30.0 | 9.1 | 12.4 | 2.3 | 4.3 | 54 | 54 | 0.14 | 1.38 | 7.57 | 8.04 | 7.12 | 7.32 |
| 434 | 0–3 | LC | Ah1 | 38.0 | 43.3 | 40.6 | 33.9 | 15.2 | 14.3 | 6.2 | 8.5 | 23 | 0 | 18.4 | 6.98 | 7.54 | 6.03 | 6.97 | 5.33 |
| | 10–20 | | Ah2 | 33.4 | 40.6 | 34.9 | 33.3 | 21.7 | 23.3 | 10.0 | 2.8 | 22 | 0 | 8.96 | 2.76 | 7.50 | 4.88 | 7.02 | 3.43 |
| | 50–70 | | Ck | 36.4 | 42.9 | 31.0 | 36.3 | 24.5 | 17.1 | 8.1 | 3.6 | 38 | 0 | 1.03 | 0.34 | 7.65 | 4.71 | 7.11 | 3.36 |

LC: Luvic chernozem; HC: Haplic chernozem; ML: Mollic leptosol; EL: Eutric leptosol; RL: Renzdic leptosol; CR: Calcaric regosol; HL: Haplic luvisol

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# STANDARDIZATION OF DOSAGE OF LIQUID AND CYST FORMULATIONS OF *Azospirillum* FOR DIFFERENT APPLICATION METHODS

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*Azospirillum* bioinoculant is well known for its high nitrogen-fixing and plant growth-promoting characters. Carrier-based bioinoculants generally suffer from shorter shelf-life, poor quality, high contamination and low field performance. As an alternative, the liquid and cyst formulations of *Azospirillum* inoculants can play a significant role. Liquid and cyst formulations of *Azospirillum* were developed by adding amendments to the NFb broth and to MSM medium, respectively, which have longer shelf-life and tolerance to adverse conditions such as temperature and desiccation. The dosage of liquid and cyst-based formulations of *Azospirillum* for various inoculation methods such as seed treatment, seedling root dipping and soil application was standardized and their survival was studied. Inoculum levels of 10 ml/kg seeds, 150 ml/quantity of seedlings required for 1 ha and 300 ml/ha were found to be the optimum doses for seed treatment, seedling root dipping and soil application methods, respectively. The liquid and cyst formulations of *Azospirillum* have exhibited better adherence and survival on seeds, seedling roots and in the rhizosphere than the carrier-based form. These results indicated that there is substantial room to improve the liquid and cyst formulations of *Azospirillum* inoculant to obtain the desired benefits.

**Key words:** *Azospirillum*, liquid and cyst formulation, dosage, application methods

## Introduction

The nitrogen-fixing rhizobacterium *Azospirillum* lives in close association with plant roots, where it exerts beneficial effects on the plant growth and yield of many crops of agronomic importance (Okon and Labandera-Gonzalez, 1994; Okon and Vanderleyden, 1997). Of the various rhizosphere-associated $N_2$-fixing bacteria, *Azospirillum* species are probably the most studied and appear to have significant potential for commercial applications. Although the potential of *Azospirillum* has been well documented and substantiated, the successful exploitation of this diazotroph is hampered by several factors. Altering the

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rhizosphere microflora by seed, root or soil inoculation with specific organisms has long been recognized as a practical possibility, but formulation inadequacies and the non-availability of good quality inoculums are often the most common barriers to the commercialization and widespread adoption of bioinoculants. FAO (1991) reported that most of the international producers of biofertilizers are engaged in the production of carrier-based inoculants. The biofertilizers manufactured in India are also carrier-based in general and suffer from short shelf-life, poor quality, high contamination and low and unpredictable field performance (Hegde, 2002). A frequent observation is that in peat-based inoculants, the number of viable cells decreases from $10^9$ to $10^7$ cfu per g after 90 days of storage (Okon and Itzigsohn, 1995).

Therefore, liquid and cyst formulations of *Azospirillum* were developed by adding amendments to NFb broth (Dobereiner and Day, 1974) and MSM medium (Neyra and Van Berkum, 1977). The liquid and cyst formulations have longer shelf-life and tolerance to adverse conditions such as temperature and desiccation. In the present study, the dosage for the liquid and cyst formulations of *Azospirillum* was standardized for the common methods of application of bioinoculants, namely seed treatment, seedling root dipping and soil application.

## Materials and methods

*Standardization of dosage for seed treatment*

To standardize the dosage of liquid and cyst formulations of *Azospirillum* for seed treatment and to assess their survival on seeds, the following treatment schedule was adopted with varied inoculum levels.

$T_1$ – Carrier-based *Azospirillum*
$T_2$ – Liquid formulation of *Azospirillum* + sterile water
$T_3$ – Liquid formulation + rice gruel
$T_4$ – Cyst-based liquid formulation of *Azospirillum* + sterile water
$T_5$ – Cyst-based liquid formulation + rice gruel

Seeds of rice (ADT-46) and maize (CO-1) were used for this study. Surface-sterilized seeds were coated with the inoculum at three levels (5 ml, 10 ml and 15 ml per kg of seed). An equal volume of sterile water or rice gruel was added with the inoculum and inoculated directly on to the seed. The carrier (lignite)-based inoculum was mixed at a rate of 20 g with 50 ml rice gruel per kg of seed to make a slurry (semi-liquid mixture of lignite-based inoculum and rice gruel) and the seeds were mixed in the slurry and kept aseptically at room temperature.

Immediately after seed inoculation with the liquid- and carrier-based inoculants, samples of 10 seeds were assayed to determine the number of viable bacterial cells on the seeds. Each seed sample was placed in a 25 ml flask containing 10 ml diluent (0.85% NaCl and 0.01% Tween 80) and vortexed for 5 minutes. The samples were serially diluted and 100 µl of $10^{-6}$–$10^{-9}$ dilutions were spread-plated on NFb agar plates. The remaining seeds were kept in Petri dishes in sealed containers at room temperature (28–30°C). The above procedure was repeated at 6, 12, 24, 36 and 48 h after inoculation and the results were expressed as cfu per seed. The germination percentage of the seeds was then determined.

*Standardization of dosage for seedling root dipping*

The following treatment schedule was adopted to standardize the dosage for the seedling root dipping method.

$T_1$ – Carrier-based *Azospirillum* + sterile water
$T_2$ – Liquid formulation of *Azospirillum*
$T_3$ – Liquid formulation + sterile water
$T_4$ – Cyst-based liquid formulation of *Azospirillum*
$T_5$ – Cyst-based liquid formulation + sterile water

Seedlings of rice (Co 47) and tomato (PKM 1) were raised in sterile soil and the roots were washed thoroughly in sterile water. The liquid and cyst formulations were inoculated at three levels (100, 150 and 200 ml per ha seedlings) in 25 l of sterile water. The carrier-based formulation was also mixed with 25 l of sterile water at a rate of 1 kg to make a slurry and the seedlings required for one ha were dipped in the slurry and kept in the shade. After 30 min dipping in the inoculum, a sample consisting of 10 seedlings was taken from each treatment and the root portions were cut and mixed thoroughly. One gram of mixed roots was transferred to $10^{-1}$ NCl and serially diluted and plated. The survival of *Azospirillum* cells at 0, 1, 2, 3 and 6 h intervals was assessed and the results were expressed as cfu per g of root on a dry weight basis.

*Standardization of dosage for soil application*

To standardize the dosage of different formulations of *Azospirillum* for soil application, the following treatment schedule was adopted with varied inoculum levels.

$T_1$ – Carrier-based *Azospirillum* + farmyard manure (FYM)
$T_2$ – Liquid formulation of *Azospirillum* + sterile water
$T_3$ – Liquid formulation + sterile water + FYM
$T_4$ – Cyst-based liquid formulation of *Azospirillum* + sterile water
$T_5$ – Cyst-based liquid formulation + sterile water + FYM

Seedlings of rice (Co 47) were raised in sterile soil and 25-day-old seedlings were carefully planted out in the pre-sterilized soil. Direct seeded maize (M + M : CO-1) was also grown under sterilized conditions. They were inoculated with the *Azospirillum* inoculum at three levels, 200, 300 and 400 ml $ha^{-1}$ mixed with 25 kg of well-powdered farmyard manure and/or 25 l of sterile water according to the treatment schedule. Carrier-based *Azospirillum* was inoculated at 2 kg $ha^{-1}$ with 25 kg of well-powdered farmyard manure. One g of rhizosphere soil sample was taken from the inoculated pots to examine the survival of *Azospirillum* at 0, 15, 30, 45 and 60 days after inoculation. The number of cells present in the rhizosphere was calculated using the serial dilution and plating technique and the results were expressed as cfu per g of soil on a moisture-free basis.

## Results and discussion

### *Dosage standardization for seed treatment*

In general, the population of *Azospirillum* adhering to the seeds was gradually reduced from 0 h to 48 h of incubation (Tables 1 and 2). The cyst formulation of *Azospirillum* performed better than liquid and carrier-based formulations. An inoculum level of 15 ml/kg of seeds resulted in the maximum population, when compared to 10 ml and 5 ml/kg of seeds, but there was no significant difference between the 15 ml and 10 ml/kg levels. In the liquid- and cyst-based formulations, a population of over $10^5$ cfu/g was observed both at the 10 ml and 15 ml inoculum levels up to 24 h after seed treatment, whereas carrier-based *Azospirillum* resulted in a population of only $10^4$ cfu/g. Singleton et al. (2002) reported that an inoculum level of 5 ml/kg seeds was optimum for the *Rhizobium* liquid formulation when tested on soybean seeds.

*Table 1*
Survival of liquid and cyst formulations of *Azospirillum* on rice seeds

| Treatments | Population of *Azospirillum* (cfu/seed) | | | | | |
|---|---|---|---|---|---|---|
| | 0 h | 6 h | 12 h | 24 h | 36 h | 48 h |
| | | | Inoculum level (5 ml/kg seed) | | | |
| $T_1$ | 7.65 | 7.57 | 6.68 | 4.53 | 2.42 | – |
| $T_2$ | 7.72 | 7.61 | 6.73 | 4.66 | 2.51 | – |
| $T_3$ | 7.73 | 7.69 | 6.79 | 4.71 | 2.56 | – |
| $T_4$ | 7.71 | 7.69 | 6.83 | 4.77 | 2.57 | – |
| $T_5$ | 7.73 | 7.70 | 6.85 | 4.79 | 2.62 | – |
| | | | Inoculum level (10 ml/kg seed) | | | |
| $T_1$ | 7.65 | 7.57 | 6.68 | 4.53 | 2.42 | – |
| $T_2$ | 8.69 | 8.62 | 6.76 | 5.69 | 2.67 | – |
| $T_3$ | 8.76 | 8.72 | 6.81 | 5.74 | 3.61 | 1.20 |
| $T_4$ | 8.76 | 8.72 | 7.73 | 6.69 | 4.62 | 2.25 |
| $T_5$ | 9.73 | 8.77 | 7.79 | 6.73 | 4.72 | 2.32 |
| | | | Inoculum level (15 ml/kg seed) | | | |
| $T_1$ | 7.65 | 7.57 | 6.68 | 4.53 | 2.42 | – |
| $T_2$ | 8.69 | 7.63 | 6.75 | 5.72 | 2.70 | – |
| $T_3$ | 8.77 | 8.73 | 6.80 | 5.75 | 3.64 | 1.25 |
| $T_4$ | 8.78 | 8.73 | 7.76 | 6.73 | 4.67 | 2.34 |
| $T_5$ | 9.75 | 8.78 | 7.79 | 6.74 | 4.74 | 2.34 |

| | SEd | CD(0.05) |
|---|---|---|
| Treatment | 0.004 | 0.008 |
| Inoculum | 0.003 | 0.006 |
| Time | 0.004 | 0.009 |
| Interaction | 0.018 | 0.035 |

Figures are $\log_{10}$ transformed values; $T_1$ – Carrier-based *Azospirillum*; $T_2$ – Liquid formulation of *Azospirillum*; $T_3$ – Liquid formulation + rice gruel; $T_4$ – Cyst formulation of *Azospirillum*; $T_5$ – Cyst formulation + rice gruel

The cyst and liquid formulations of *Azospirillum* performed better than the carrier-based one, possibly due to the prolonged survival of cells, as influenced by the addition of cell protectants and other chemicals. It appears that there is a large interaction between the strain and the medium components in determining cell numbers in the inoculant and their degree of survival after inoculation on to seeds (Singleton et al., 2002). The use of rice gruel as an adhesive enhanced the adherence of *Azospirillum* to the seed. The inoculation of liquid formulation with rice gruel favoured the adherence of a larger number of cells on the seeds, probably due to its sticky nature and nutrient content (Kundu and Gaur, 1981). A similar trend was observed for germination percentage data (not shown). Liquid inoculants can support cell survival on the seed better than the solid carrier formulation, which could have been the reason for better germination.

*Table 2*
Survival of liquid and cyst formulations of *Azospirillum* on maize seeds

| Treatments | Population of *Azospirillum* (cfu/seed) | | | | | |
|---|---|---|---|---|---|---|
| | 0 h | 6 h | 12 h | 24 h | 36 h | 48 h |
| | | | Inoculum level (5 ml/kg seed) | | | |
| $T_1$ | 7.70 | 7.63 | 6.71 | 4.60 | 2.46 | – |
| $T_2$ | 7.75 | 7.62 | 6.73 | 4.68 | 2.52 | – |
| $T_3$ | 7.77 | 5.39 | 6.78 | 4.73 | 2.60 | – |
| $T_4$ | 7.80 | 7.73 | 6.79 | 4.76 | 2.59 | – |
| $T_5$ | 7.81 | 7.76 | 6.81 | 4.78 | 2.65 | – |
| | | | Inoculum level (10 ml/kg seed) | | | |
| $T_1$ | 7.70 | 7.63 | 6.71 | 4.60 | 2.46 | – |
| $T_2$ | 8.72 | 8.68 | 6.72 | 5.69 | 3.64 | 1.25 |
| $T_3$ | 8.77 | 8.73 | 7.69 | 5.76 | 4.57 | 1.41 |
| $T_4$ | 8.74 | 8.72 | 7.73 | 6.64 | 4.64 | 2.14 |
| $T_5$ | 9.71 | 8.74 | 7.78 | 6.75 | 4.71 | 2.17 |
| | | | Inoculum level (15 ml/kg seed) | | | |
| $T_1$ | 7.70 | 7.63 | 6.71 | 4.60 | 2.46 | – |
| $T_2$ | 8.73 | 8.69 | 6.76 | 5.71 | 3.68 | 1.27 |
| $T_3$ | 8.78 | 8.73 | 7.71 | 5.73 | 4.62 | 1.46 |
| $T_4$ | 8.76 | 8.75 | 7.72 | 6.66 | 4.65 | 2.00 |
| $T_5$ | 9.73 | 8.76 | 7.80 | 6.77 | 4.73 | 2.25 |

| | SEd | CD (0.05) |
|---|---|---|
| Treatment | 0.081 | 0.161 |
| Inoculum | 0.063 | 0.125 |
| Time | 0.089 | 0.177 |
| Interaction | 0.347 | 0.686 |

Figures are $\log_{10}$ transformed values; $T_1$ – Carrier–based *Azospirillum*;$T_2$ – Liquid formulation of *Azospirillum*; $T_3$ – Liquid formulation + rice gruel; $T_4$ – Cyst formulation of *Azospirillum*; $T_5$ – Cyst formulation + rice gruel

*Dosage standardization for seedling root dipping*

The survival of *Azospirillum* on the roots of seedlings was gradually reduced from 0 h to 6 h of incubation. The cyst formulation of *Azospirillum* at 200 ml/ha with sterile water led to the highest survival of cells on the seedlings, followed by the liquid formulation and carrier-based *Azospirillum* (Table 3, Fig. 1). The cyst formulation of *Azospirillum* at 150 ml/ha with sterile water was found to be on par with 200 ml/ha of cyst formulation inoculated alone. Carrier-based *Azospirillum* at an inoculum level of 100 ml/ha resulted in the minimum survival and adherence of cells on the seedlings. Liquid formulations contain a high count range from $10^8$ to $10^{10}$ cfu per ml, which ensures a maximum number of effective bacteria for action (Krishna Chandra et al., 2005). The enhanced survival of cyst inoculant on the roots of seedlings may be attributed to the fact that this formulation helped a larger number of cells to adhere to the seedlings and maintained a high population of *Azospirillum* for a long time. The cyst cells may rapidly regenerate into vegetative cells immediately after they come in contact with the seedlings. Thus, the cyst-based liquid formulation favoured the prolonged survival of *Azospirillum* on the roots of seedlings, when compared to the other types of inoculants.

*Table 3*
Survival of liquid and cyst formulations of *Azospirillum* on the roots of rice seedlings

| Treatments | Population of *Azospirillum* (cfu/g root on a dry wt. basis) | | | | |
|---|---|---|---|---|---|
| | 0 h | 1 h | 2 h | 3h | 6 h |
| | | | Inoculum level (100 ml/ha) | | |
| $T_1$ | 7.71 | 7.68 | 6.55 | 4.71 | 3.65 |
| $T_2$ | 7.76 | 7.69 | 6.64 | 4.76 | 3.67 |
| $T_3$ | 8.73 | 7.76 | 6.72 | 4.79 | 3.70 |
| $T_4$ | 8.74 | 7.78 | 6.73 | 5.61 | 3.73 |
| $T_5$ | 8.78 | 8.71 | 6.82 | 5.72 | 3.75 |
| | | | Inoculum level (150 ml/ha) | | |
| $T_1$ | 7.71 | 7.68 | 6.55 | 4.71 | 3.65 |
| $T_2$ | 8.74 | 7.77 | 6.69 | 4.77 | 4.65 |
| $T_3$ | 8.77 | 8.69 | 6.77 | 5.68 | 4.69 |
| $T_4$ | 9.67 | 8.79 | 6.77 | 5.70 | 4.73 |
| $T_5$ | 9.70 | 8.79 | 6.79 | 5.76 | 4.75 |
| | | | Inoculum level (200 ml/ha) | | |
| $T_1$ | 7.71 | 7.68 | 6.55 | 4.71 | 3.65 |
| $T_2$ | 8.74 | 7.79 | 6.71 | 5.64 | 4.68 |
| $T_3$ | 8.76 | 8.68 | 6.76 | 5.73 | 7.71 |
| $T_4$ | 9.69 | 8.80 | 6.78 | 5.74 | 4.75 |
| $T_5$ | 9.71 | 8.81 | 7.62 | 5.76 | 4.77 |

| | SEd | CD (0.05) |
|---|---|---|
| Treatment | 0.003 | 0.007 |
| Inoculum | 0.002 | 0.005 |
| Time | 0.003 | 0.007 |
| Interaction | 0.014 | 0.028 |

Figures are $\log_{10}$ transformed values; $T_1$ – Carrier-based *Azospirillum* + sterile water; $T_2$ – Liquid formulation of *Azospirillum*; $T_3$ – Liquid formulation + sterile water; $T_4$ – Cyst formulation of *Azospirillum*; $T_5$ – Cyst formulation + sterile water

*Dosage standardization for soil application*

The application of bioinoculants either directly to the soil or to the seeds is the simplest method of inoculation, which was used in numerous greenhouse and field experiments (Sharma and Manjula, 2005; Sharief et al., 2006). The population of inoculated *Azospirillum* gradually increased from 0 days after transplanting (DAT) to 60 DAT. The maximum population was recorded in the treatment inoculated with the cyst formulation of *Azospirillum* at 400 ml/ha with sterile water and farmyard manure and the minimum population for the carrier-based *Azospirillum* inoculated with farmyard manure (Table 4, Fig. 2). The 400 ml/ha inoculum level performed better than 300 or 200 ml/ha in the rhizosphere of both rice and maize. However, the inoculum level of 300 ml/ha was statistically on par with 400 ml/ha.

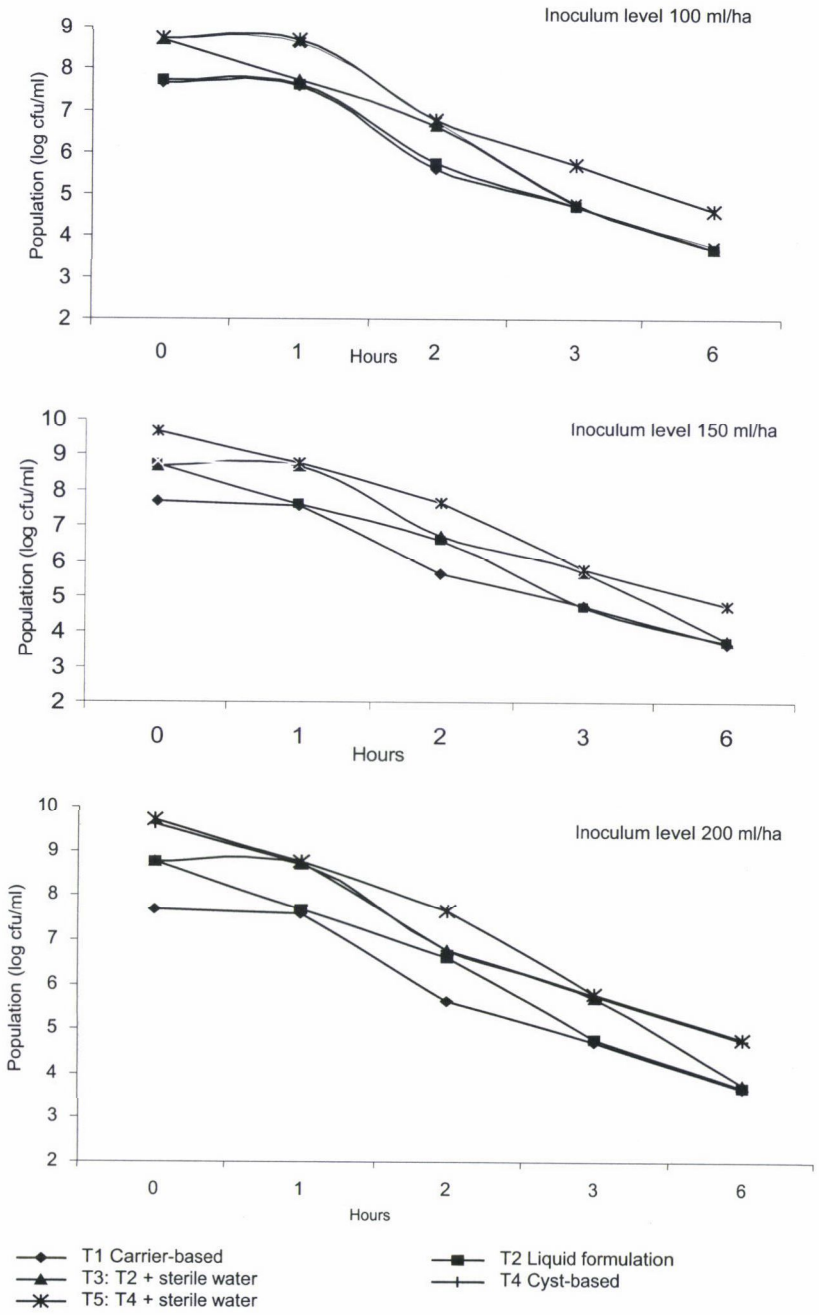



*Fig. 1.* Survival of liquid and cyst formulations of *Azospirillum* on tomato seedlings

*Table 4*
Survival of liquid and cyst formulations of *Azospirillum* in the maize rhizosphere

| Treatments | Population of *Azospirillum* (cfu/g) | | | | |
|---|---|---|---|---|---|
| | 0 DAT | 15 DAT | 30 DAT | 45 DAT | 60 DAT |
| | | | Inoculum level (200 ml/ha) | | |
| $T_1$ | 4.62 | 6.71 | 7.60 | 7.98 | 8.73 |
| $T_2$ | 5.73 | 7.63 | 7.85 | 8.02 | 8.86 |
| $T_3$ | 4.45 | 7.74 | 7.92 | 8.73 | 8.91 |
| $T_4$ | 5.81 | 7.78 | 7.95 | 8.09 | 8.93 |
| $T_5$ | 5.83 | 7.86 | 8.01 | 8.79 | 8.96 |
| | | | Inoculum level (300 ml/ha) | | |
| $T_1$ | 4.62 | 6.71 | 7.60 | 7.98 | 8.73 |
| $T_2$ | 5.72 | 7.76 | 7.88 | 8.73 | 8.88 |
| $T_3$ | 5.77 | 7.79 | 7.97 | 8.79 | 8.92 |
| $T_4$ | 6.65 | 7.85 | 8.02 | 8.86 | 9.35 |
| $T_5$ | 6.72 | 7.91 | 8.06 | 8.91 | 9.43 |
| | | | Inoculum level (400 ml/ha) | | |
| $T_1$ | 4.62 | 6.71 | 7.60 | 7.98 | 8.73 |
| $T_2$ | 5.74 | 7.77 | 7.90 | 8.76 | 8.89 |
| $T_3$ | 5.77 | 7.80 | 7.97 | 8.79 | 8.94 |
| $T_4$ | 6.65 | 7.84 | 8.03 | 8.87 | 9.39 |
| $T_5$ | 6.73 | 7.91 | 8.06 | 8.93 | 9.47 |

| | SEd | CD (0.05) |
|---|---|---|
| Treatment | 0.056 | 0.111 |
| Inoculum | 0.043 | 0.086 |
| Days | 0.056 | 0.111 |
| Interaction | 0.218 | 0.431 |

Figures are $\log_{10}$ transformed values; $T_1$ – Carrier-based *Azospirillum* + farmyard manure;$T_2$ – Liquid formulation + sterile water; $T_3$ – Liquid formulation + sterile water + farmyard manure; $T_4$ – Cyst formulation + sterile water; $T_5$ – Cyst formulation + sterile water + farmyard manure

Bashan et al. (1995) reported that *Azospirillum* survived well in the rhizosphere soils tested, regardless of soil type, bacterial strain or origin of the soil. Higher *Azospirillum* survival was found in treatments inoculated with the cyst formulation, possibly due to the fact that the encystations allowed the organisms to survive under varied soil conditions (Sadasivan and Neyra, 1987). Soria et al. (2006) suggested that the physiological manipulation of cultures improved tolerance of stress conditions. The cell protectants added in the liquid formulation appeared to protect the cells from the effect of desiccation by slowing the drying rate. The addition of farmyard manure enhanced the survival of *Azospirillum* in the rhizosphere, as evidenced in treatments inoculated with the liquid and cyst formulations. These results indicated that there is substantial room to improve the liquid and cyst formulations of *Azospirillum* inoculant to obtain the desired benefits.

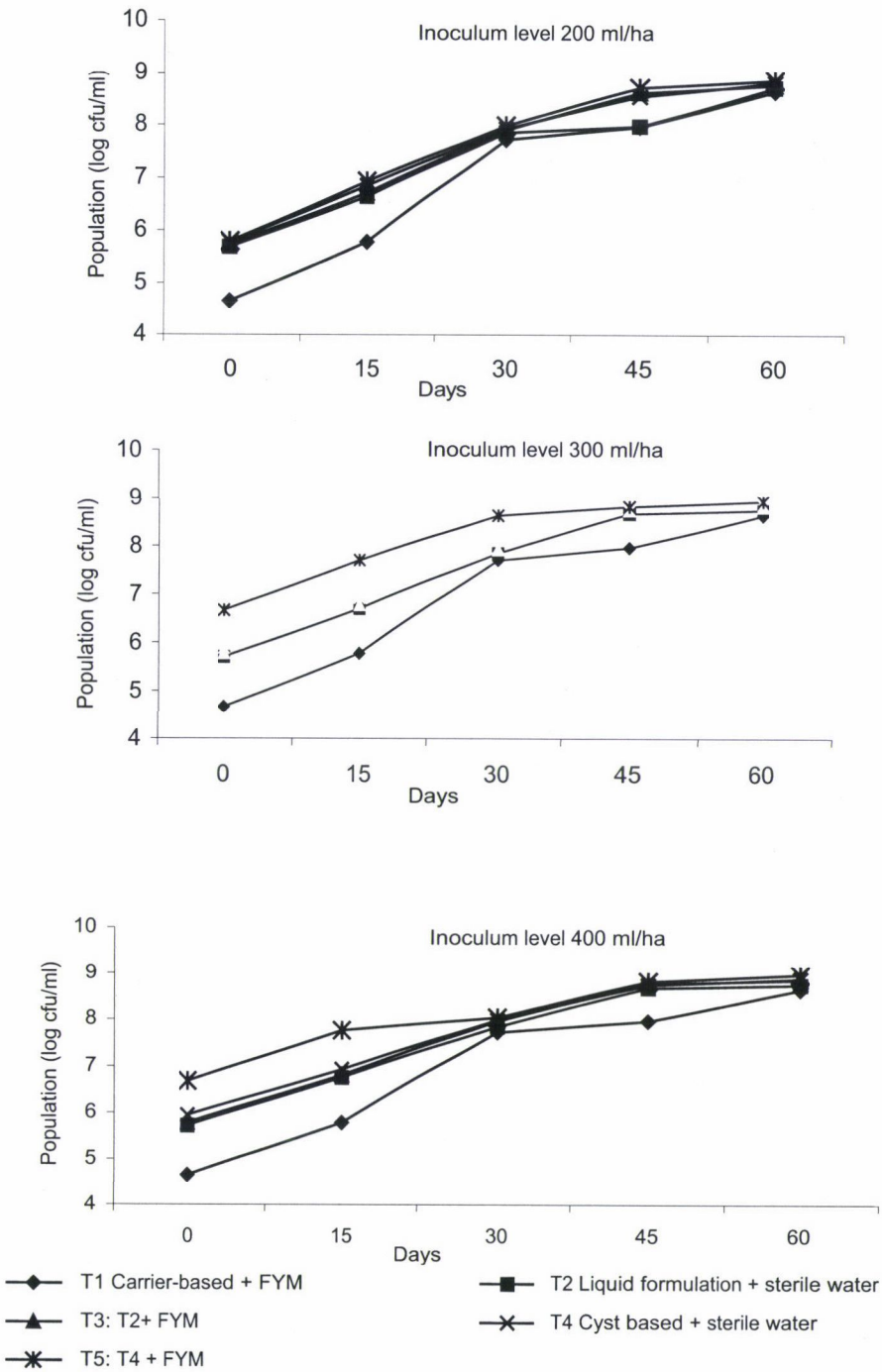



*Fig. 2.* Survival of liquid and cyst formulations of *Azospirillum* in the rice rhizosphere

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*Short communication*

# INFLUENCE OF OIL CONTENT ON THE EQUILIBRIUM MOISTURE CONTENT OF EVENING PRIMROSE (*Oenothera* SPP.) SEEDS

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Seed moisture content is a well-recognised index of safe storage. However, when in equilibrium with the storage environment it is merely an indicator of the relative humidity of the air, which is the primary regulator of the growth of moulds and insects. The relationship is influenced by the profile of the seed components. During the 1990s, significant increases in the seed oil content of evening primrose were achieved through plant breeding. This paper shows that the equilibrium moisture content of evening primrose seeds declines significantly with increasing oil content. Hence, the moisture isotherm is altered and newer cultivars must be stored at slightly lower seed moisture contents to ensure that seed and oil quality are maintained.

**Key words**: evening primrose, *Oenothera* spp., seed, oil content, moisture content, relative humidity

## Introduction

Evening primrose (*Oenothera* spp.) has been grown as a high-value oilseed crop in several countries in western and central Europe, North America and Australasia. The seed oil contains gamma linolenic acid (GLA), an uncommon fatty acid which is used in pharmaceuticals and nutritional supplements (Horrobin, 1992) and which must therefore comply with strict quality criteria. Evening primrose seeds are very small, dark brown and somewhat irregular and angular in shape. The seeds and their components, and methods for their analysis, are described by Hudson (1984) and Christie (1999).

Seed quality is influenced by pre-harvest as well as post-harvest handling. As with oilseed rape, evening primrose crops are not left to ripen unaided but are either swathed or desiccated prior to combine-harvesting (Simpson and Fieldsend, 1993). In New Zealand, an important evening primrose seed production area, seeds are dried after harvest to a storage moisture content of 9.5–10% using driers with a maximum drying temperature of 30°C.

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The maintenance of both seed and oil quality, through factors such as germination capacity and seed vigour, fungal infestation, acid and peroxide values, is dependent on keeping the seeds dry during storage. Over a period of time, the moisture content of the seeds will reach equilibrium with their storage environment, characterised by the relative humidity (RH) of the atmosphere and temperature. It has long been known (e.g. Wilson, 1994) that seeds of different crop species have different equilibrium moisture contents ($MC_e$), determined by several factors, including properties of the seed coat and the profile of the seed constituents, of which lipids are the most hydrophobic.

Fieldsend (2007) reported that in evening primrose substantial increases in both the oil content in the seeds and the GLA content of the oil have been achieved through plant breeding. In overwintered crops harvested in New Zealand, seed oil content increased from approximately 26.5% to approximately 28.5% as the older cultivars Paul and Peter were replaced by Merlin and then Rigel during the period 1990–1997, whilst the equivalent figures for spring-sown crops were 25% and 28%. Using seeds of different breeding lines harvested from replicated yield trials, this study investigated whether significantly different $MC_e$ between seeds of different evening primrose cultivars can occur as a consequence of differences in seed oil contents.

## Materials and methods

Two randomised complete block yield trials, one overwintered (trial W) and one spring-sown (trial S), each with four replications, were established in August 1994 and April 1995, respectively, on the trialground of Scotia Pharmaceuticals Ltd., located near Chelmsford, Essex, UK (latitude 51° 47'N, longitude 0° 31'E, altitude 50 m). Trial W was swathed with a Haldrup plot swather on 16 August and was combine-harvested with a Wintersteiger Nursery Master Elite combine-harvester on 14 September 1995. Trial S was swathed on 3 October and combine-harvested on 1 December 1995 using the same equipment. In both cases, 15–20 g seed samples were taken ex-combine to record the moisture content at harvest using the method described below. The combine-harvested bulks were dried in cotton bags on a forced-air drier using unheated air and the dry seeds were screen cleaned and winnowed to remove trash.

Sub-samples of cleaned seeds for analysis were transferred to paper packets in early December (trial W) or mid-December (trial S) and were stored under room conditions. To determine $MC_e$, 5 g of seeds was taken from each packet and placed in weighing boats. The seeds from each replication were placed in an open plastic box which was in turn placed inside an airtight bag. All the bags were kept under room conditions for seven days. The seeds were then weighed prior to the determination of moisture content on a wet weight basis by drying in an oven at 103°C for 17 hours in compliance with International Seed Testing Association rules.

Thousand-seed weight (TSW) was measured by weighing 200 whole seeds per sample with a top-pan balance accurate to 0.001 g. Seed oil content was determined using an OAI Newport 4000 NMR analyser (Fieldsend and Morison, 2000).

## Results

*Overwintered trial W*

At the time of combine-harvesting, the seed moisture contents of the cultivars Peter, Merlin and Rigel were 11.8%, 11.1% and 11.1%, respectively. On 8 December their $MC_e$ were 9.4%, 9.0% and 8.9%, respectively (Fig. 1). There was a significant correlation ($r^2 = -0.731$, $p < 0.01$) between seed oil content and $MC_e$ (Fig. 1). ANOVA of $MC_e$ data gave a LSD of 0.202 ($p < 0.05$) indicating that, for example, the $MC_e$ for the cultivars Merlin and Rigel, which had seed oil contents of 27.7% and 28.0%, respectively, were significantly lower than that of Peter, where the seed oil content was 26.6%. The TSW of the 12 entries ranged from 0.36 g for Merlin and one other breeding line to 0.47 g for Rigel. There was no correlation between TSW and $MC_e$ ($r^2 = 0.090$, ns; data not shown).

The seed samples of the three cultivars which had been dried to 0% moisture content were kept in a low-humidity seed store with a RH of approximately 47% and a temperature of approximately 18°C from mid-December 1995 to mid-January 1996, when they were returned to room conditions. On 14 February 1996, these were analysed for $MC_e$. The $MC_e$ of Peter was significantly higher than those of Merlin and Rigel (Fig. 2) (LSD = 0.080, $p < 0.05$).

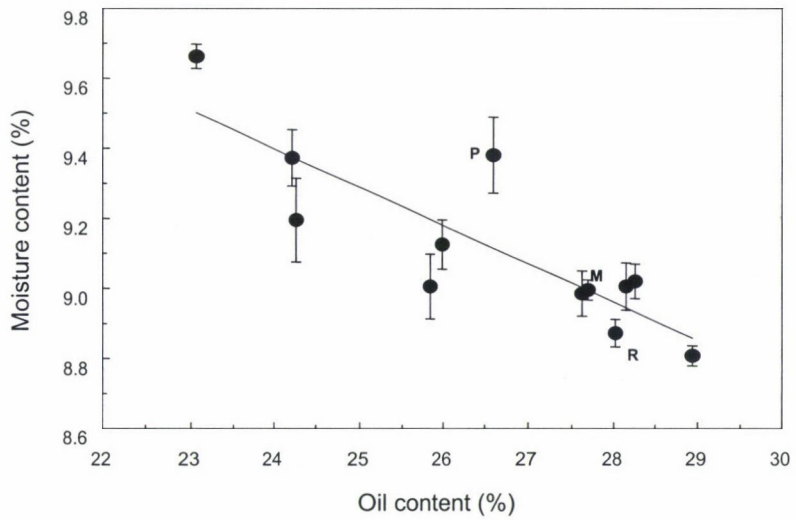



*Fig. 1.* Relationship between seed equilibrium moisture content and oil content of 12 cultivars and breeding lines harvested from overwintered trial W, measured on 8 December 1995 after post-harvest storage under room conditions. The relationship is described by the linear regression equation $y = -0.1111x + 12.073$. P = Peter, M = Merlin and R = Rigel. Error bars represent ± 1 SE.

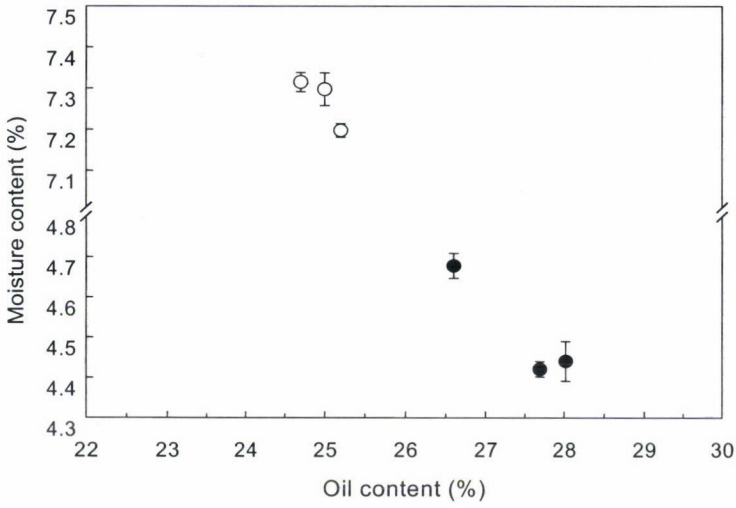



*Fig. 2.* Relationship between seed equilibrium moisture content and oil content, measured on 14 February 1996, of three cultivars harvested from overwintered trial W, after oven drying to 0% moisture on 8 December 1995 (closed symbols) and three cultivars harvested from spring-sown trial S, measured on 14 February 1996 after a post-harvest period of storage at low relative humidity (approximately 47%) followed by storage under room conditions (open symbols). Error bars represent ± 1 SE

*Spring-sown trial S*

The moisture content of the combine-harvested seed samples was very high (21.2% for Vulcan and 23.4% for Merlin) but by 20 December, after cleaning and drying on the seed drier, the moisture contents of Vulcan, Merlin and Rigel had declined to 10.7%, 10.5% and 10.2%, respectively. The seeds were kept in the low-humidity seed store until mid-January 2006, when they were transferred to room conditions. $MC_e$ were measured on 14 February 1996 and an inverse relationship between oil content and $MC_e$ was indicated (Fig. 2). The $MC_e$ of Merlin was significantly lower than that of Vulcan (LSD = 0.112, $p < 0.05$). Again, no correlation between TSW and moisture content was observed (data not shown).

## Discussion

The seed oil contents in this study covered a similar range to those reported by Fieldsend (2007) and, in line with his results, seeds harvested from trial W had higher oil contents than seeds harvested from trial S. The $MC_e$ of evening primrose seeds is strongly influenced by oil content. This applies to seed ex-

combine, to seed allowed to equilibrate under room conditions and to seed taking up moisture after having been dried to 0%. The very low $MC_e$ values obtained from the latter are probably a manifestation of hysteresis (Wilson, 1994). If the regression equation from Figure 1 is applied to the data of Fieldsend (2007), whereas seeds from overwintered crops harvested in 1990 may have had an $MC_e$ of approximately 9.1%, those harvested in 1995–1997 would have had, under the same storage conditions, an $MC_e$ of 8.9%. For spring-sown crops, $MC_e$ would have declined over the same period from approximately 9.3% to 8.9%.

The important factor in determining seed quality during storage, however, is not seed moisture content, but the amount of water in the air surrounding the seeds which, when in equilibrium with the water in the seeds, is described as the equilibrium relative humidity ($RH_e$, Wilson, 1994). With changes in seed oil content the moisture isotherm will be altered such that at a given $RH_e$ and air temperature, seeds with higher oil contents will have lower $MC_e$ values. It is conceivable, therefore, that a $MC_e$ which is known to indicate that the $RH_e$ is below the threshold for the safe long-term storage of seeds of older cultivars is, for newer cultivars, equivalent to a $RH_e$ which is above it.

These data indicate the need to ensure that crop management methods are constantly updated for crops that are undergoing rapid development, such as those that are new to agriculture. In the case of evening primrose, seeds of newer cultivars with higher oil contents must be stored at slightly lower moisture contents to ensure that both seed and oil quality are maintained.

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*Review*

# ROLE OF S-METHYLMETHIONINE IN THE PLANT METABOLISM

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S-methylmethionine (SMM), a naturally occurring, biologically active compound, is a free amino acid derivative, which is increasingly recognised as playing an important part in the plant metabolism. SMM, which is synthesised from methionine, is involved in crucial processes in the S metabolism, such as the regulation of methionine and S-adenosyl methionine levels, the methylation processes taking place in cells, and the transport and storage of sulphur in certain phases of development. It is of great importance in the development of resistance to abiotic and biotic stress factors, as it is a direct precursor in the biosynthesis of the osmoprotectants and other S-containing compounds involved in defence mechanisms, while also influencing the biosynthesis of major plant hormones such as polyamines and ethylene. The present paper discusses our increasing understanding of the role played by SMM in the plant metabolism and its possible role in the improvement of traits that enable plants to overcome stress.

**Key words**: methionine, S-methylmethionine (SMM), plant methabolism

## Introduction

S-methylmethionine (SMM) is a naturally occurring, biologically active compound. It is a non-proteinogenic free amino acid [$(CH_3)_2$-S-$(CH_2)_2$-$CH(NH_2)$-COOH], which is becoming increasingly recognised as an important component in the plant S metabolism. In plants SMM was first detected in cabbage, which accumulates large quantities of the compound (McRorie et al., 1954), but was later demonstrated in numerous other higher plants (Williams and Nelson, 1974; Giovanelli et al., 1980; Schwenn et al., 1983; Dufour, 1986; Macnicol, 1986; Macnicol and Randall, 1987). Its occurrence in the plant kingdom is now thought to be general.

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An increasing body of data has been published in recent years on the functions of SMM, showing that it is synthesised from methionine (Met), plays a role in the regulation of Met and S-adenosyl-methionine (AdoMet) levels (Pimenta et al., 1998; Hacham et al., 2002; Kocsis et al., 2003), is involved in the methylation processes taking place in cells (Ranocha et al., 2000) and is an important compound for the transport and storage of sulphur (Bourgis et al., 1999). It is capable of mitigating the effect of numerous abiotic and biotic stressors through the production of the osmoprotectant dimethyl-sulphoniopropionate (Trossat et al., 1998; Kocsis et al., 1998) and by enhancing the biosynthesis of polyamines via the down-regulating of ethylene production (Lásztity et al., 1992; Kissimon et al., 1994; Gyetvai et al., 2002; Rácz et al., 1996). Reaction pathways linked to SMM are responsible for selenium tolerance in certain plants, thus allowing these species to be used to remove selenium from soils and water bodies (de Souza et al., 2000; Tagmount, 2002). The complexity of its functions indicates that SMM plays a wide-ranging regulatory role in plant physiological processes, the most important of which will be outlined below. It should be noted here that SMM has also been produced synthetically and is widely used in human medicine and in veterinary practice. It has a beneficial effect in the treatment of intestinal ulcers and in the after-care of intestinal infections, also being used in livestock farming as a feed additive (Kopinski et al., 2007; Augspurger et al., 2005). It is commonly known as vitamin U.

## The SMM cycle

The synthesis of SMM from Met by methylation has been confirmed in many species by monitoring the Met metabolism. When the roots absorbed isotopically labelled Met (labelled sulphur or methyl groups) or sulphate, the radioactive signal could also be detected in SMM in the plants (Splittstoesser and Mazelis, 1967; Bauer and Yang, 1972; Datko et al., 1978; Schilling and Kende, 1979; Mudd and Datko, 1986). SMM accumulation was also observed in mutants over-producing methionine (Curtiss et al., 1987; Inaba et al., 1994; Gakiere et al., 2002; Hacham et al., 2007). Other studies using isotopic labelling demonstrated that SMM was able to revert to Met through the methylation of homocysteine, thus creating an SMM cycle (Fig. 1). Hanson and Kende (1976), Mudd and Datko (1990) and Ranocha et al. (2001) demonstrated the presence of a complete SMM cycle *in vivo* in the species *Ipomoea tricolor* and *Lemna paucicostata* and in various organs of *Arabidopsis*. Less attention has been given, however, to the reaction in which SMM is decomposed to dimethyl-sulphide (DMS) and homoserine by the SMM hydrolase enzyme (Giovanelli et al., 1980).

The synthesis of SMM from methionine was first studied in oak in a cell-free medium (Sato et al., 1958). The enzyme catalysing the irreversible reaction is S-adenosyl-methionine:methionine S-methyltransferase (EC 2.1.1.12, MMT)

(Giovanelli et al., 1980). MMT is specific for L-methionine and accepts S-adenosyl-methionine (AdoMet) as methyl donor (Greene and Davis, 1960). MMT is a homotetramer consisting of a 115-kDa subunit and was isolated first from *Wollastonia biflora* (James et al., 1995a) and later from barley (Pimenta et al., 1998). It can be clearly distinguished on the basis of its size from other methyltransferases, such as that methylating protein methionine or those specific for other substrates. Under *in vivo* conditions optimum enzyme activity requires a pH of 6–7 and a temperature of 50°C (Dethier et al., 1991; James et al., 1995a). The presence of MMT has been demonstrated in numerous species and in various plant organs, both on the basis of its specific enzyme activity and via the antibodies that recognise the protein (Karr et al., 1967; Giovanelli et al., 1980; James et al., 1995a; Pimenta et al., 1998; Ranocha et al., 2001). Similar studies proved that the MMT enzyme could be found in the cytoplasm (Trossat et al., 1996). In *Arabidopsis* and maize, MMT is present in the genome as a single-copy gene (Bourgis et al., 1999; Ranocha et al., 2001). By sequencing the cDNA of *W. biflora* MMT, Bourgis et al. (1999) were able to identify the sequences of *Arabidopsis* and maize MMT from databases. The analysis of the sequences demonstrated a section consisting of 300 amino acids at the N terminal, exhibiting similarity to the conservative regions of methyltransferases. A region responsible for the binding of AdoMet was also found in this section. No signal sequences responsible for organellar transport were detected at the N terminal, confirming the cytoplasmic localisation of the enzyme. At the C terminal a section with 400 amino acid residues exhibited similarity with the α family of proteins that bind pyridoxal-5'-phosphate (PLP). The MMT enzyme, however, is unlikely to bind PLP due to the lack of Lys, the most important amino acid for binding, so the region probably has a regulatory role.

The other member of the SMM cycle is S-methyl-methionine:homocysteine S-methyltransferase (EC 2.1.1.10, HMT), which catalyses the production of 2 mol methionine from 1 mol SMM and 1 mol homocysteine. The activity of the HMT enzyme was first detected by Turner and Shapiro (1961) in the seeds of various plant species. Sequence analyses identified three single-copy HMT genes in the genome of *Arabidopsis* (AtHMT-1, -2, -3), while four were found in maize (ZmHMT-1, -2, -3, -4) (Ranocha et al., 2001). Functional enzymes were found to be transcribed from all three HMT genes of *Arabidopsis* (Ranocha et al., 2000; 2001). So far only one enzyme gene with HMT activity has been identified in broccoli (BoHMT-1), which exhibits 92.4% homogeneity with the amino acid sequence of the AtHMT-1 gene (Lyi et al., 2007). The level of homogeneity between the HMT genes at the amino acid sequence level is 55–64% for *Arabidopsis* and 52–94% for maize, while there is 51–65% homogeneity between the HMT genes of the two species. Based on the single HMT gene found in bacteria, Ranocha et al. (2000) hypothesised that the two HMT genes found in yeasts and the multiple HMT genes found in plants evolved from a single ancestral HMT gene. Within the plant kingdom, judging

from the pedigree of the HMT genes in *Arabidopsis* and maize, two branches can be distinguished (Ranocha et al., 2001), one of which consists of the HMT-1 genes of both species, while within the other branch the monocot and dicot HMT genes have followed different evolutionary pathways (AtHMT-2, -3, ZmHMT-2, -3, -4). The HMT genes characteristically contain a zinc-finger motif, while the N terminal exhibits significant similarity to other methyltransferases. On the basis of the missing signal sequence they can be assumed to be localised in the cytoplasm. The proteins transcribed from the HMT genes are 36–38 kDa in size and the enzyme is active in the monomer form (Ranocha et al., 2000; 2001). They have a pH optimum of 7.3–7.9 and a temperature optimum of 60°C (Abrahamson and Shapiro, 1965; Dodd and Cossins, 1970; Ranocha et al., 2000). They have different levels of sensitivity to Met, which inhibits the AtHMT-2 and -3 enzymes, but not AtHMT-1. The main methyl donor of the HMT genes is SMM, though AdoMet was also observed to be used to some extent. Under *in vivo* conditions, however, this is likely to be insignificant compared to SMM (Ranocha et al., 2000). Ranocha et al. (2001) examined the activity and gene expression of the HMT enzymes of *Arabidopsis* and maize in various plant organs. In the case of *Arabidopsis* the greatest enzyme activity was observed in the roots, mainly due to the intense expression of AtHMT-1 and -2, while AtHMT-3 was more strongly expressed in the leaves. In the case of maize, ZmHMT-4 was expressed exclusively in the seeds and ZmHMT-1 partly in the flowers, but mainly in the seeds. The expression of ZmHMT-2 and -3 was also more pronounced in the seeds. The BoHMT-1 gene identified in broccoli was expressed in the roots, flowers and young leaves, but not in older leaves (Lyi et al., 2007). These differences in the expression of the HMT enzymes over time and space and in their sensitivity to Met is indicative of the complex role and effects of the SMM cycle.

## SMM and the role of the SMM cycle

An analysis of the components of the SMM cycle makes it clear that this metabolic process is ubiquitous in the plant kingdom, while the presence of homologous MMT and HMT EST genes in bryophytes suggests that the cycle is an ancient plant characteristic (Ranocha et al., 2001). The physiological role of the cycle is less clear, however, since – being a cycle – it does not change the net quantity of S or of the methyl groups. Data recorded for *Lemna*, however, reveal that the rate of SMM synthesis (320 pmol/frond × doubling) and its conversion to Met (310 pmol/frond × doubling) is far from negligible (Mudd and Datko, 1990). The importance of SMM and of the SMM cycle is demonstrated by the fact that the functioning of the cycle requires a total of 1 mol ATP (Fig. 1), so it must perform a function that justifies an energy investment of this size (Mudd and Datko, 1990). The role played by SMM in plants has not yet been clarified, but investigations have suggested a number of possibilities.

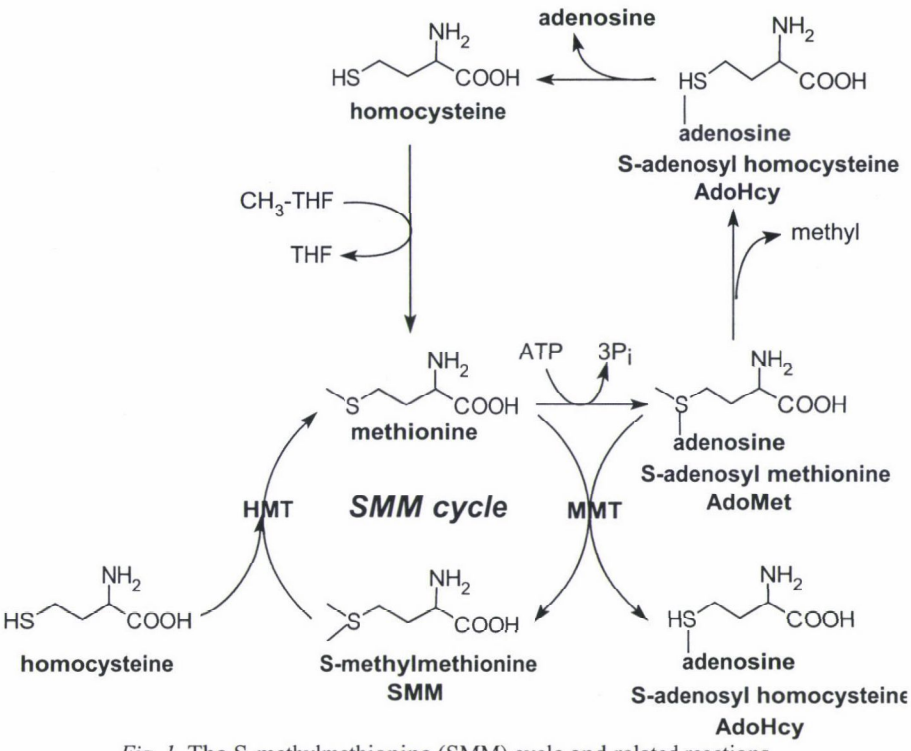



*Fig. 1.* The S-methylmethionine (SMM) cycle and related reactions. (MMT = S-adenosylmethionine:methionine S-methyltransferase; HMT = S-methyl methionine:homocysteine S-methyltransferase)

*Role of the SMM cycle in the regulation of Met and AdoMet levels*

Mudd and Datko (1990) were the first to suggest that the SMM cycle could play an important role in the regulation of the Met level if the balance began to shift in favour of AdoMet. A reduction in the Met level would allow AdoMet to revert to Met more rapidly (in two reactions), thus ensuring the free Met level required for uninterrupted protein synthesis. The significance of this aspect of the SMM cycle was analysed by Ranocha et al. (2001) using a computer model to simulate the Met metabolism in fully developed leaves of *Arabidopsis*. Data obtained by labelling Met with S isotope were used in the model, together with measurements of the quantities of the individual components in plants. If the whole of the SMM cycle was eliminated, the model predicted a substantial accumulation of AdoMet. To achieve a more detailed analysis of the theory of Mudd and Datko (1990), the model was re-programmed so that the whole of the Met quantity was converted to AdoMet. Two scenarios were then examined, one with a completely functional SMM cycle, the other with the SMM cycle eliminated and the synthesis of AdoMet retarded. In both cases the Met level was quickly restored, while the restoration of the AdoMet

level took place more rapidly when the SMM cycle was intact. It thus became clear that the SMM cycle does not regulate the free Met level. The reduction in the level of metabolically active, free Met was overcome by the release of Met from proteins and stored reserves. On the other hand, the model clearly showed that the SMM cycle was responsible for determining the AdoMet level.

Kocsis et al. (2003) provided empirical proof that the SMM cycle did not influence the Met level, but regulated that of AdoMet. The effect of the SMM cycle and the long-term effect of SMM throughout plant development was studied on *Arabidopsis* and maize mutants unable to produce SMM due to a mutation in the MMT gene, which meant that the MMT enzyme was dysfunctional and the SMM cycle was absent. In both mutants, labelled Met was incorporated into the proteins at the same rate as in the wild type, i.e. the free Met quantity did not change as the result of the mutation. In the case of the MMT-mutant *Arabidopsis* the Met level was also measured directly and was found to be the same as in the wild type. This indicated that the long-term absence of the SMM cycle caused no disruption in the free Met level in a stress-free environment. At the same time, the influence of the SMM cycle on the AdoMet level is demonstrated by the fact that the AdoMet level was higher in the MMT-mutant *Arabidopsis* than in the wild type. Further proof of this role of SMM was obtained from transgenic tobacco over-expressing one of the enzymes involved in Met synthesis, the cystathionine γ-synthase of *Arabidopsis* (Gakiere et al., 2002). In these plants the Met and SMM levels rose, but the AdoMet level did not change compared with that of the wild type, indicating that SMM has a decisive role in maintaining the AdoMet level. This is of special importance for the plant metabolism, which lacks the negative feedback present in other eukaryotes, by means of which AdoMet inhibits its own synthesis (Schröder, 1997).

*Role of the methyl group in SMM*

The structure of SMM is such that it could well act as a general methyl donor in living organisms. In addition to the methylation of homocysteine, it may be involved in other methylation processes. This was confirmed by an experiment in which the incorporation of labelled methyl groups into various meristem tissues was monitored during the transition to the reproductive phase in morning glory (Hanson and Kende, 1976). It was found that, although SMM was not converted to Met in the early phases, it was involved in methylation processes, indicating that it must have reacted with other methyl acceptors apart from homocysteine. It was observed that selenocysteine-methyltransferase (SMT), the enzyme that catalyses the selenium metabolism, accepted SMM as a methyl donor as well as AdoMet (Neuhierl et al., 1999). In addition, Sato et al. (1958) demonstrated the participation of SMM in the methylation of pectins and protopectins in oak, though its role in these processes was not significant. When the methyl group of SMM was isotopically labelled and traced in *Lemna* (Mudd and Datko, 1990), it was first found to appear in Met, and only later could the

isotopic signal be detected in components such as phosphatidyl-choline, neutral lipids, etc. whose methyl group is actually derived from Met. In *Arabidopsis* and maize mutants with no functional MMT gene and thus no SMM synthesis (Kocsis et al., 2003) the lack of SMM did not cause disturbances in the methylation processes at the plant development level, suggesting that it cannot be a general or exclusive methyl donor. Apart from direct methylation reactions, however, SMM could play some other role in the metabolism of the methyl group. In the MMT-mutant plants mentioned above (Kocsis et al., 2003) the absence of SMM resulted in a 45% rise in the methylation rate, i.e. in the AdoMet/S-adenosyl homocysteine (AdoHcy) ratio. This could be attributed to the higher level of AdoMet and lower level of AdoHcy compared to the wild type. AdoMet is the main methyl source for transmethylation processes; AdoMet-dependent methyltransferases are involved in the methylation of proteins, DNA, mRNA, pectin, sterol, ethanolamine, lignin monomers, phenols and osmolites, and thus have an important role in the metabolism, development and stress tolerance of plants (Moffatt and Weretilnyk, 2001). The function of methyltransferases is inhibited by AdoHcy, so the activity of methylation processes is determined by the AdoMet/AdoHcy ratio, which the above-mentioned experiments suggest is regulated in part by SMM. Giovanelli et al. (1980) hypothesised that the SMM cycle makes it possible to store methyl groups, so that if their *de novo* synthesis declines compared with that of homocysteine, SMM can methylate homocysteine to restore the Met level, and consequently that of its derivative AdoMet, thus ensuring the necessary quantity of methyl donors.

*Role of SMM in the transport and storage of S; shifts in the SMM cycle over time and space*

The role of SMM in the transportation of S was detected in experiments on fully developed wheat in the grain-ripening stage (Bourgis et al., 1999). Met containing labelled S was introduced into the leaves, after which the S transport forms were examined in phloem exudate collected from the stem section close to the spike with the help of aphids. Some 80% of the total quantity of S-containing components consisted of SMM, which thus far exceeded the quantity of glutathione, a compound previously proved to play a role in transport processes. On these grounds Bourgis et al. (1999) suggested that, although the whole SMM cycle can be completed in all the plant organs, there may be a shift in the ratio of the two reactions making up the cycle. SMM synthesis may be more active in the leaves, providing SMM for transport via the phloem. When this reaches the maturing grain, the SMM cycle shifts towards the conversion of SMM into Met. The role of SMM in S transport was confirmed in numerous other plant species, in which the presence of SMM was demonstrated in the phloem exudate.

There is a characteristic time-shift in the SMM cycle, with a change in the SMM level, in the course of germination, suggesting that SMM could also play a

role in the transport and storage of S in seedlings. In the case of wheat, for example, HMT proved to be more active in dry seeds and at the beginning of germination, while in later stages of germination MMT exhibited greater activity (Allamong and Abrahamson, 1977). In germinating barley the initially low SMM level did not begin to rise until the 4$^{th}$ day (Dethier et al., 1991), while the MMT enzyme exhibited an increase in activity from the 3$^{rd}$–4$^{th}$ day (Pimenta et al., 1998). The immunohistochemical analysis of germinating barley revealed that the MMT enzyme was active in the endosperm and in the aleuron cells (Pimenta et al., 1998). It is thus hypothesised that the Met produced by the decomposition of storage proteins in these organs is converted by the MMT enzyme into SMM, which is able to migrate to other parts of the germ, where it is converted back into Met, allowing it to take part in protein synthesis. The diverse properties of the various HMTs present in a given plant indicate that different HMTs may play important roles in different plant parts and in different phases of plant development. For instance, an extremely Met-insensitive HMT was found in jack bean (Abrahamson and Shapiro, 1965) and in pea seeds (Dodd and Cossins, 1970), while the Met-insensitive AtHMT-2 was also principally expressed during the germination of *Arabidopsis* (Ranocha et al., 2000). The absence of Met inhibition also means that Met can be accumulated to a greater extent in the seed than in other plant organs (Giovanelli et al., 1980; Ranocha et al., 2000).

*Role of SMM in the synthesis of dimethyl-sulphoniopropionate (DMSP)*

High concentrations of dimethyl-sulphoniopropionate (DMSP) have been found chiefly in algae (Stefels, 2000). It is only found to a limited extent in the plant kingdom, though sugarcane and certain members of the salt-tolerant genera *Spartina* and *Wollastonia* were reported to accumulate large quantities (Dacey et al., 1987; Otte et al., 2004; Paquet et al., 1995). Plant experiments have suggested a number of possible functions for DMSP, but further research will be required to confirm these functions and determine their exact mechanisms. DMSP is involved in methylation processes in higher plants and plays a role in deterring herbivores. It is decomposed to acrylate and the volatile compound dimethyl sulphide (DMS), which gives a decisive flavour to certain foodstuffs and, when entering the atmosphere, plays an important role in atmospheric processes and in the global S cycle (Bentley and Chasteen, 2004). The fact that DMSP can be converted back into Met means that it can function as a S store. Experiments conducted mostly on algae suggest it has a cryoprotectant and antioxidant role, while it appears to act as an osmoprotectant in higher plants, particularly in salt-tolerant plants that accumulate DMSP. Its structure confirms the possibility of its involvement in osmoregulation, as it bears a resemblance to glycine-betaine, also known as an osmoprotectant (McNeil et al., 1999).

The synthesis of DMSP in higher plants may take place along two pathways (Fig. 2) (Hanson et al., 1994). In *Wollastonia biflora*, a member of the

*Compositeae*, DMSP is synthesised from SMM via DMSP-aldehyde (James et al., 1995b), while in *Spartina alterniflora*, a member of the *Gramineae*, synthesis takes place via DMSP-amine (Kocsis et al., 1998). This indicates that the pathways of DMSP synthesis developed independently in these two evolutionarily distant families. The site of the various steps in DMSP synthesis in the cell was examined in *W. biflora* by Trossat et al. (1996) using enzyme activity measurements and immunostaining following cell fractionation. It was confirmed that the MMT enzyme responsible for SMM synthesis was to be found in the cytoplasm, but the DMSP-aldehyde dehydrogenase (DDH) enzyme that catalyses the DMSP-aldehyde → DMSP reaction was localised in the chloroplasts. The DMSP production of intact chloroplasts incubated with SMM proved that the SMM synthesised in the cytoplasm is transported to the chloroplasts, where it is converted first to DMSP-aldehyde and then to DMSP. The ability of plants to accumulate DMSP is thus largely dependent on the SMM-transporting efficiency of the chloroplasts. Trossat et al. (1998) found that in plants that do not accumulate DMSP only 1–15% of the total SMM content was located in the chloroplasts, while in the case of *W. biflora*, which accumulates DMSP, this figure was 40%. In response to salt stress the SMM content of the chloroplast rose even further in *W. biflora*, to 80% of the total SMM. This change was reflected by a rise in the DMSP level, thus ensuring the osmotic protection of the processes taking place in the chloroplasts.

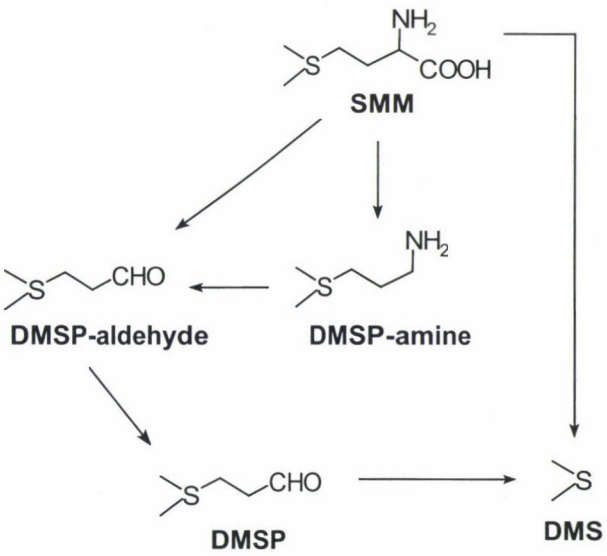



*Fig. 2.* Synthesis of dimethylsulphoniopropionate and dimethyl-sulphide.
(SMM = S-methylmethionine; DMSP-aldehyde = dimethylsulphoniopropionaldehyde; DMSP-amine = dimethylsulphoniopropylamine; DMSP = dimethylsulphoniopropionate; DMS = dimethyl sulphide)

*Role of SMM in selenium phytoremediation*

Selenium (Se) is an essential microelement in livestock feeding and in the human diet, but in large quantities it is toxic, so phytoremediation techniques, involving the use of Se-tolerant plants, are being widely investigated for the cleaning of soils and water bodies contaminated with Se. It has not yet been confirmed that Se is essential for plants, but in plants too the toxic effect is caused by the fact that its chemical properties are similar to that of S, allowing it to follow the S metabolic pathway and to use the transporters and enzymes of this pathway to become incorporated into methionine and cysteine to form selenomethionine (SeMet) and selenocysteine (SeCys). When these are used in protein synthesis they cause anomalies in protein functions. Se-tolerant plants are capable of avoiding the toxic effects of Se and are able to accumulate or remove Se in large quantities, making them suitable for use in phytoremediation. SMM and the enzymes involved in the SMM metabolism all participate in the detoxification of SeMet and SeCys.

The removal of SeMet takes place via conversion to dimethylselenide (DMSe), a harmless volatile compound (Ellis and Salt, 2003). In the course of this reaction SeMet is converted to selenomethylmethionine (SeMM) (Fig. 3). It was proved by Tagmount et al. (2002) that the catalyser of this reaction was the MMT enzyme, which is able to methylate not only Met but also SeMet, thus synthesising SeMM. In an *Arabidopsis* mutant where the MMT enzyme was dysfunctional there was a substantial reduction in the emission of volatile Se, while the incorporation of the MMT gene from *Arabidopsis* into *E. coli*, which possesses no MMT enzyme, resulted in the emission of volatile Se. This experiment also demonstrated that in Se-tolerant plants where the defence mechanism is not based on Se accumulation, Se emission plays a role in tolerance. In accordance with this, the Se tolerance of the MMT-mutant *Arabidopsis* decreased. The further conversion of SeMM to volatile DMSe is possible by means of two pathways, either via dimethyl selenopropionate (DMSeP) or through the direct decomposition of SeMM, catalysed by the methylmethionine-hydrolase enzyme (Lewis et al., 1974). The formation of DMSe from DMSeP was confirmed by de Souza et al. (2000). When various Se forms were added (selenate, selenite, SeMet, DMSeP), DMSeP was found to result in the highest level of volatile Se emission. The enzymes involved in the process are assumed to be the same as those participating in the synthesis of DMSP and DMS, but few data are available on these in plants. The clarification of the metabolic pathways involved in the synthesis of non-toxic, volatile Se and the stimulation of their functioning in plants would provide an extremely useful, economical tool for the phytoremediatory removal of Se pollutants from the soil.

The other major pathway of Se tolerance is the accumulation and transformation of methylselenocysteine (MSeCys). In the course of detoxification, SeCys is converted to MSeCys in a methylation reaction (Fig. 3), i.e. the creation of a non-proteinogenic amino acid prevents its incorporation into

proteins and allows the plant to accumulate or transform the non-toxic, methylated derivative. The synthesis of MSeCys is of interest not only for phytoremediation, but also in medicine, due to its anti-cancer effect. Its synthesis is catalysed by the selenocysteine-methyltransferase enzyme (SMT), the over-expression of which leads to the enhancement of Se tolerance and the accumulation of MSeCys (Ellis et al., 2004; LeDuc et al., 2004). One important property of the SMT enzyme from the point of view of the SMM metabolism is its acceptance of SMM as a methyl donor, as well as AdoMet (Neuhierl et al., 1999).

*Protective effect of SMM against cold stress*

In addition to its role in osmotic stress, by enhancing the synthesis of the osmoprotectant DMSP as outlined above, SMM may also play an outstanding part in overcoming the damaging effects of cold stress. This was suggested by the characteristically high SMM content of members of the Brassicaceae family, which are known to have good frost resistance. The accumulation of SMM could thus have a role in resistance to cold stress (Gyetvai et al., 2002; Rácz et al., 2007). In cold-sensitive plants, SMM treatment may lead to an increase in resistance. For example, an improvement in photosynthetic activity was recorded in cold-treated maize when SMM was applied (Kissimon et al., 1994). The favourable effect of SMM was also demonstrated by studies in which the increased permeability of membranes and the consequent ion leakage from the cells was investigated in response to cold stress. In both the leaves and roots of pea, maize, and wheat varieties with various levels of cold tolerance, the uptake of SMM from the nutrient solution provided protection from the membrane-damaging effect of cold stress, as there was no increase in ion leakage due to cold stress after SMM treatment (Lásztity et al., 1997; Gyetvai et al., 2002; Szalai et al., 2003; Rácz et al., 2007). Three possible mechanisms for the protective effect of SMM against cold stress were suggested by Gyetvai et al. (2002).

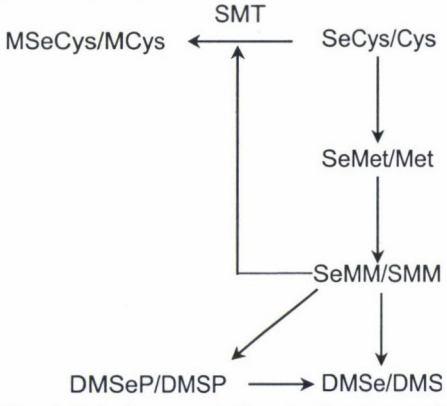



*Fig. 3.* Selenium metabolism in Se-tolerant plants
(MSeCys/MCys = methyl-selenocysteine/methylcysteine; SMT = selenocysteine-methyltransferase; SeCys/Cys = selenocysteine/cysteine; SeMet/Met = selenomethionine/methionine; SeMM/SMM = selenomethylmethionine/S-methylmethionine; DMSeP/DMSP = dimethyl-selenopropionate/dimethyl-sulphopropionate; DMSe/DMS = dimethylselenide/dimethyl sulphide)

On the one hand, the stimulation of DMSP synthesis from SMM could maintain a satisfactory osmotic state in the cells, while secondly an increase in the formation of DMS, which has radical-scavenging properties, could reduce damage to the membranes. In addition, SMM may also exert its effect via the polyamines, the synthesis of which has been found to be influenced by SMM. The external application of SMM led to an increase in the levels of various polyamines (putrescine, agmatine, spermidine) in many plants (maize, wheat, pea, soybeans), though it had no influence on the spermine level (Lásztity et al., 1997; Rácz et al., 2007). The role of SMM in the synthesis of polyamines may be related in part to the regulation of the AdoMet level, since the decarboxylated derivative of AdoMet supplies the propylamino group involved in the formation of spermidine from putrescine and of spermine from spermidine (Fig. 4). However, new light was shed on the role of SMM in polyamine synthesis by studies in which SMM-induced polyamine synthesis was examined after the specific inhibition of AdoMet decarboxylation. The presence or absence of the inhibitor did not influence the spermidine and spermine levels recorded after SMM treatment, i.e. SMM was responsible for a polyamine synthesis pathway independent of decarboxylated AdoMet (Lásztity et al., 1992; Gyetvai et al., 2002; Rácz et al., 2007). In this pathway the propylamino group is provided directly by SMM for the synthesis of spermidine and spermine, while the enhancement of the agmatine and putrescine levels is ensured by its influence on other processes. The wide-ranging effect of polyamines on plant life processes also accounts for the complex role of SMM, and the ability of polyamines to stabilise membranes, proteins and DNA may also be related to the mechanism by which SMM overcomes stress (Galston and Kaur-Sawhney, 1995; Bouchereau et al., 1999).

*Effect of SMM on ethylene production*

The effect of SMM in regulating the AdoMet level allows it to participate in metabolic pathways starting from AdoMet. In this connection the effect of SMM on methylation processes and polyamine synthesis have already been discussed, but the SMM content of plants also has an influence on ethylene synthesis, which also starts from AdoMet via Met. The effect exerted on ethylene production is, however, complex. It stems partly from the competition between polyamine biosynthesis and ethylene biosynthesis for AdoMet, an intermediate compound common to both processes, and partly from the fact that polyamines inhibit ethylene synthesis at several points (the transformation of AdoMet to 1-aminocyclopropane-1-carboxylate, ACC, and that of ACC to Met). At the same time, ethylene also has an inhibitory effect on polyamine synthesis. Results yet to be published indicated a lower level of ethylene production after SMM treatment. However, in transgenic tobacco over-expressing *Arabidopsis* cystathionine γ-synthase, an enzyme involved in Met synthesis, but without the regulating region at the N terminal, higher levels of free Met, Met present in dissolved protein and SMM were observed compared to the wild type,

accompanied by 40 times the normal level of ethylene production. A clear understanding is also complicated by the fact that the functioning of the 1-aminocyclopropane-1-carboxylate (ACC) synthase enzyme responsible for ethylene synthesis is inhibited by SMM itself under *in vitro* conditions, though this is not necessarily the case in plants (Ko et al., 2004).

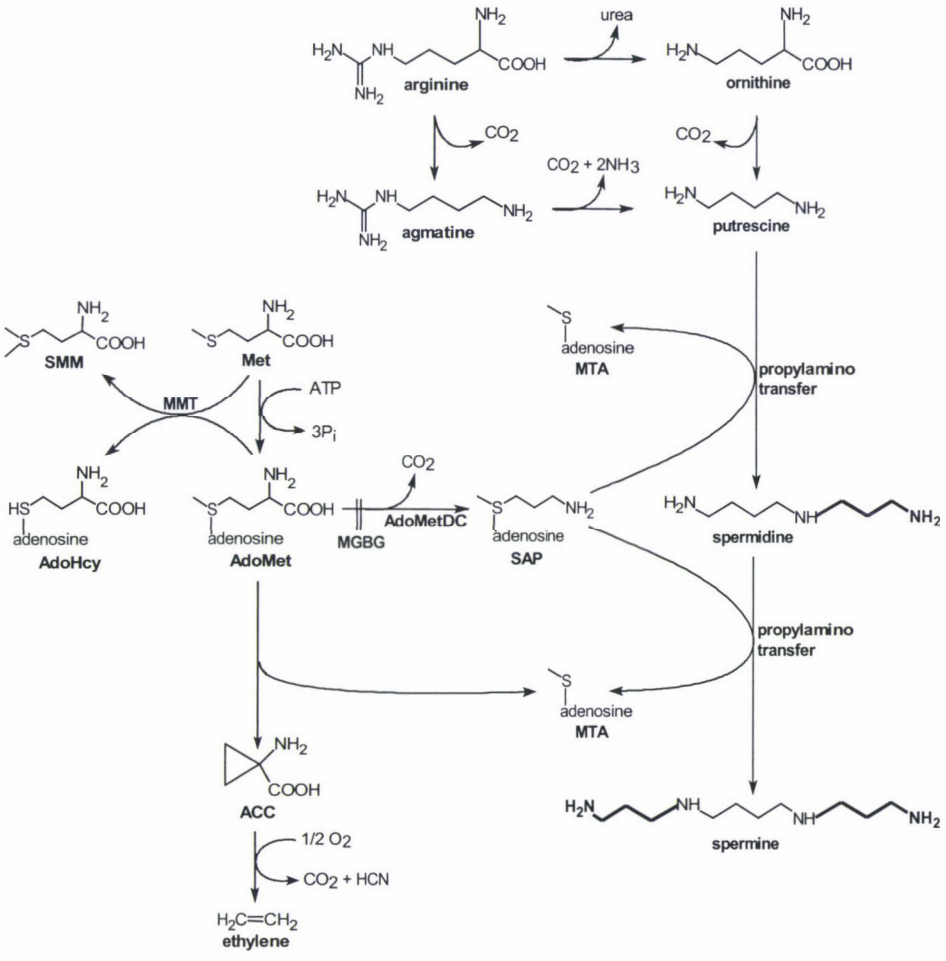



*Fig. 4.* Biosynthesis of polyamines and ethylene. In the formula of spermidine and spermine the propylamino group is printed in bold. (SMM = S-methylmethionine; Met = methionine; AdoHcy = S-adenosyl homocysteine; AdoMet = S-adenosyl methionine; MMT = S-adenosylmethionine:methionine S-methyltransferase; MGBG = methylglyoxal-bis-guanyl hydrazone; AdoMetDC = AdoMet decarboxylase; SAP = S-adenosyl-methylthiopropylamine; MTA = 5'-methylthioadenosine; ACC = 1-aminocyclopropane-1-carboxylate)

## Summary

On the basis of the functions outlined above, SMM exerts an effect at numerous points in the plant metabolism (Fig. 5), thus influencing most stages in the life of the plants. Since it was first identified, it has been found to play a role in an increasing number of processes. The complexity of its functions is a clear indication of its ability to influence key components in the metabolism, thus exerting an effect on the S metabolism, methylation processes and the production of hormones, resulting in numerous favourable physiological effects (germination and growth stimulation, enhancement of photosynthetic activity, protein and nucleic acid synthesis) and in protection against stress factors (drought, low temperature) via the production of metabolites (e.g. anthocyanines, carotenoids) involved in biotic and abiotic stress responses (Rácz I., Lásztity D., personal communication). As the compounds arising during the S metabolism, known collectively as sulphur-containing defence compounds (SDCs), are increasingly considered to play an important role in the improvement of defence potential (Rausch and Wachter, 2005), new results can be expected on the effect of SMM in these processes. Both SMM itself and its products are only accumulated in a few plant groups, so these play an emphatic role in studies on the SMM metabolism. The favourable effect of this compound on the plant metabolism and its ability to protect plants from stress suggest that SMM could have a valuable economic role.

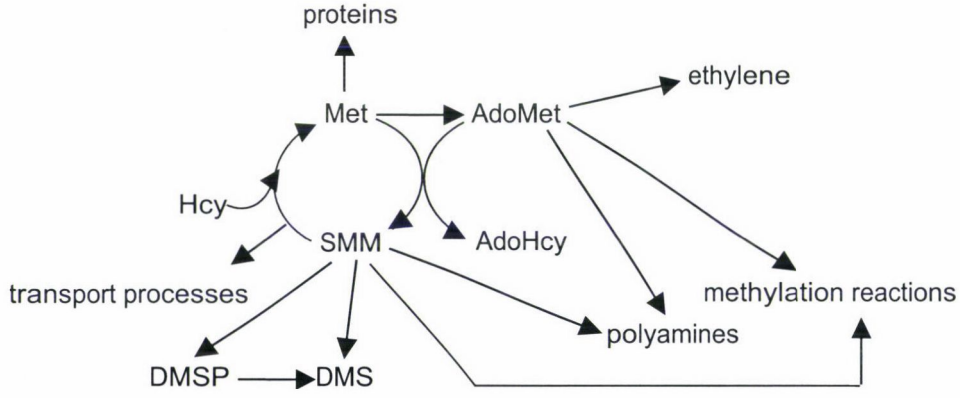



*Fig. 5.* Functions of S-methylmethionine in the plant metabolism.
Met = methionine; AdoMet = S-adenosyl methionine; Hcy = homocysteine; SMM = S-methylmethionine; AdoHcy = S-adenosyl homocysteine; DMSP = dimethylsulphoniopropionate; DMS = dimethyl-sulphide

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ORIGINAL PAPERS

Effect of temperature and radiation on photosynthesis productivity in chestnut populations (*Castanea sativa* Mill. cv. Judia)
*P. Almeida, L.-T. Dinis, J. Coutinho, T. Pinto, R. Anjos, J. Ferreira-Cardoso, M. Pimentel-Pereira, F. Peixoto* and *J. Gomes-Laranjo* ..... 193

Morphological attributes of oriental spruce [*Picea orientalis* (L.) Link.] seedlings grown in peat-based media amended with natural zeolite
*S. Ayan* and *F. Tilki* ..... 363

Isolated microspore cultures of a Hungarian durum wheat (*Triticum turgidum* L.) cultivar, Martondur 1
*F. Bakos, A. Fábián* and *B. Barnabás* ..... 157

Study of the effect of plant density on the growth of maize (*Zea mays* L.) hybrids using the Richards function
*Z. Berzsenyi* and *Q. L. Dang* ..... 417

Effects of *Glomus mosseae* strains of different origin on plant macro- and micronutrient uptake in Cd-polluted and unpolluted soils
*I. Biró* and *T. Takács* ..... 183

Comparison of different methods used in calculating the effect of stripe rust on wheat grain yields
*N. Bolat* and *F. Altay* ..... 89

Rutin content of the grain of buckwheat (*Fagopyrum esculentum* Moench. and *Fagopyrum tataricum* Gaertn.) varieties grown in southern Italy
*A. Brunori* and *G. Végvári* ..... 265

Maize population hybrids: Successful genetic resources for breeding programs and potential alternatives to single-cross hybrids*
*M. J. Carena* ..... 27

Participatory sorghum varietal evaluation and selection in Pakistan*
*S. R. Chughtai, J. Fateh, M. H. Munawwar* and *M. Hussain* ..... 19

Effect of integrated nutrient management in a soybean–wheat crop sequence on the yield, micronutrient uptake and post-harvest availability of micronutrients on Typic Ustochrepts soil
*S. K. Dadhich* and *L. L. Somani* ..... 205

Properties of humic and fulvic acids under the long-term use of fertilizers in a rice-wheat-cowpea system on a Mollisol
*D. K. Das* and *Nand Ram* ..... 293

Rheological properties of wheat dough in some hexaploid Indian wheat varieties
*K. Gajalakshmi* and *V. R. K. Reddy* ..... 235

The papers indicated by an asterisk were presented orally at the 20th International Conference of the EUCARPIA Maize and Sorghum Section, held in Budapest, Hungary on 20–24 June, 2006.

Incorporation of leaf rust resistance genes into winter wheat genotypes using marker-assisted selection
*M. Gál, G. Vida, A. Uhrin, Z. Bedő* and *O. Veisz* ..... 149

Correlations and path analysis of yield components in synthetic varieties of sunflower (*Helianthus annuus* L.)
*A. T. Göksoy* and *Z. M. Turan* ..... 339

Soil changes after forty years of succession in an abandoned coppice in the Czech Republic
*R. Hédl* and *K. Rejšek* ..... 453

Chemical quality parameters of maize hybrids in various FAO maturity groups as correlated with yield and yield components
*Z. Hegyi, I. Pók, C. Szőke* and *J. Pintér* ..... 217

Quality of maize (*Zea mays* L.) kernels as affected by the NP supplies of the soil
*Z. Izsáki* ..... 99

Competition, light quality and seedling growth of Russian wildrye grass *(Psathyrostachys juncea)*
*P. G. Jefferson* and *R. Muri* ..... 49

Effect of combinations of light intensity and photoperiod on heading date of barley (*Hordeum vulgare* L.)
*I. Karsai, K. Mészáros, B. Kőszegi, Z. Bedő* and *O. Veisz* ..... 397

Sulphur management in moong (*Phaseolus aureus* L.) and raya (*Brassica juncea* L.) crops
*M. P. S. Khurana* and *R. L. Bansal* ..... 437

Yield of switchgrass *(Panicum virgatum* L.*)* as influenced by cutting management
*Z. Kiss, A. F. Fieldsend* and *D. D. Wolf* ..... 227

Effect of different sowing densities on some characteristics of *Isatis tinctoria* L. and *Isatis constricta* Davis and on the recovery of indican
*S. Kizil, N. Arslan* and *K. M. Khawar* ..... 251

Pyrophosphate:fructose 6-phosphate 1-phosphotransferase is involved in the mobilization of sugar reserves in the taproots of cold- and drought-stressed carrot plants
*G. Kovács, S. Sorvari, P. Scott* and *O. Toldi* ..... 71

Effects of acid treatment and composting on bones used as fertilizer
*D. Kovács, G. Kardos* and *G. Füleky* ..... 115

Gene system governing stomatal number and specific leaf weight in bread wheat (*Triticum aestivum* L.)
*A. Kumar* and *S. C. Sharma* ..... 299

Importance of plant growth-promoting rhizobacteria in enhancing the seed germination and growth of watermelon attacked by fungal pathogens
*S. Lokesh, B. G. Bharath, V. B. Raghavendra* and *M. Govindappa* ..... 243

RAPD variation of late-maturing sorghum [*Sorghum bicolor* (L.) Moench] landraces from Ethiopia
*S. Mamo, A. Ayana* and *T. Tesso* ..... 375

Comparison of some methods for estimating vegetation periods in maize*
*C. L. Marton, L. Kálmán, T. Árendás, P. Bónis* and *D. Szieberth* ..... 1

Effect of 1-MCP treatment on apple biochemical content and physiological disorders
*U. Moor, K. Karp, P. Põldma* and *M. Starast* ..... 61

Kinetics of cadmium in different Indian and Hungarian soils: Incubation study at field capacity
*B. S. Panwar, M. S. Grewal* and *L. Márton* ..... 165

Effect of increased UV-B radiation on the anthocyanin content of maize (*Zea mays* L.) leaves*
*J. Pintér, E. Kósa, G. Hadi, Z. Hegyi, T. Spitkó, Z. Tóth, Z. Szigeti, E. Páldi* and *L. C. Marton* ..... 7

Effects of post-emergent herbicides on *Trichoderma harzianum,* a potential biocontrol agent against *Sclerotinia sclerotiorum* in soybean cropping
*M. C. Rollán, C. Mónaco, G. Lampugnani, N. Arteta, D. Bayo* and *M. I. Urrutia* .... 355

Effect of reduced tillage on quality traits of soybean [*Glycine max* (L.) Merr.]
*M. Sabo, D. Jug* and *I. Jug* ..... 83

Electrophoretic and immunochemical techniques for the molecular reassessment of relationships within *Vicieae*
*R. H. Sammour* ..... 131

*In vitro* and *in vivo* screening for cold tolerance in winter × spring wheat-derived doubled haploids following the wheat × maize system
*S. Sharma* and *H. K. Chaudhary* ..... 273

Effect of residue recycling on a cropping system based on fodder sorghum
*E. Shekinah, A. Alagesan, C. Jayanthi* and *N. Sankaran* ..... 347

Performance and gene effects for root characters and micronutrient uptake in wheat inoculated with arbuscular mycorrhizal fungi and *Azotobacter chroococcum*
*R. Singh, R. K. Behl, P. Jain, N. Narula* and *K. P. Singh* ..... 325

Correlation between maize genotypes and the stalk rot caused by maize *Fusarium*
*C. Szőke, T. Árendás, F. Rácz, J. Pintér, E. Nagy* and *C. L. Marton* ..... 447

Protective effect of two synthetic compounds against chlorsulfuron injury in maize (*Zea mays* L.)
*G. Stoilkova* and *P. Yonova* ..... 283

Evaluation of blue-green algal inoculation on specific soil parameters
*K. Swarnalakshmi, D. W. Dhar* and *P. K. Singh* ..... 307

The papers indicated by an asterisk were presented orally at the 20th International Conference of the EUCARPIA Maize and Sorghum Section, held in Budapest, Hungary on 20–24 June, 2006.

Standardization of dosage of liquid and cyst formulations of *Azospirillum* for different application methods
*R. Thamizh Vendan* and *M. Thangaraju* ..... 475

Quality responses of winter wheat cultivars to nitrogen and fungicide applications in Croatia
*B. Varga, Z. Svečnjak, Z. Jurković* and *M. Pospišil* ..... 37

Productivity and nitrogen use efficiency parameters in cotton cultivars with varying N levels and soil types under rainfed conditions
*M. V. Venugopalan, K. B. Hebbar, P. Tiwary, S. Chatterji, V. Ramamurthy, O. Challa* and *B. A. Sonune* ..... 383

Co-inoculation of nitrogen-fixing and phosphate-solubilizing bacteria to promote growth, yield and nutrient uptake in chickpea
*P. A. Wani, M. S. Khan* and *A. Zaidi* ..... 315

Comparison of germination responses of cultivated wheat (*Triticum*) and its wild relative (*Aegilops*) species under salinity, temperature and light
*M. Yildiz* and *E. Kasap* ..... 407

Assessment of genotype × environment interactions for yield and morphine content in opium poppy (*Papaver somniferum* L.)
*H. K. Yadav, S. Shukla* and *S. P. Singh* ..... 331

Effects of nitrogen fertilisation and two growing sites on biomass production and lignan content of linseed (*Linum usitatissimum* L.): Second year
*R. Zimmermann, U. Bauermann* and *C. Spedding* ..... 173

SHORT COMMUNICATIONS

Influence of oil content on the equilibrium moisture content of evening primrose (*Oenothera* spp.) seeds
*A. F. Fieldsend* ..... 485

Correlation analysis of yields and related physiological variables in twelve generations of durum wheat
*S. N. Sharma* and *Y. Sharma* ..... 125

Estimates of variation and heritability of some quantitative and quality characters in *Triticum turgidum* L. ssp. *durum* (Desf.)
*S. N. Sharma* and *Y. Sharma* ..... 261

REVIEW

Role of S-methylmethionine in the plant metabolism
*D. Szegő, E. Kósa* and *E. Horváth* ..... 491

OBITUARY ..... 393

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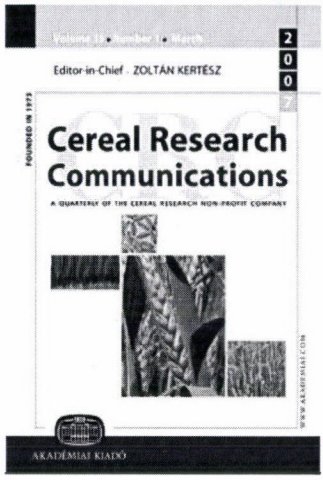
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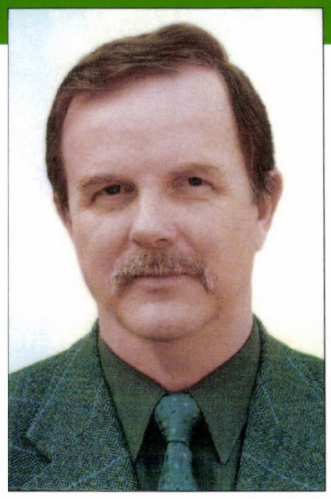
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