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Effect of Kinetin on Lesion Development and Infection Sites in Xanthi-nc Tobacco Infected by TMV: Single-cell Local Lesions

By

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Both the crude and the ultracentrifuged extracts of the kinetin-treated Xanthi-nc tobacco leaf tissues were more infective than was expected on the basis of the number of local lesions caused by TMV. In fact, infectivity of 100 lesions from the kinetin-treated half leaves exceeded that of 100 lesions from the control halves. The infective RNA content of the kinetin-treated half leaves did not change in spite of the significant reduction in lesion number. The virus did not become systemic as a result of kinetin treatment. By selective staining of necrotic cells in tissues with Evans blue it was shown that the actual number of local lesions was not reduced as a result of kinetin treatment. This treatment suppresses only the number of necroses visible to the naked eyes, but more necrotic single cells were seen on the kinetin-treated half leaves under the microscope.

It was shown by several investigators that cytokinins, such as kinetin or benzyladenine, may influence virus infection as well as virus multiplication (SELMAN, 1964; KIRÁLY and POZSÁR, 1964; KIRÁLY and SZIRMAI, 1964; DAFT, 1965; ALDWINCKLE and SELMAN, 1967; MILO and SRIVASTAVA, 1969; KIRÁLY *et al.*, 1968; ALDWINCKLE, 1975).

The results from several laboratories seem to be rather contradictory concerning the effect of cytokinins on lesion number in local lesion hosts and on virus infectivity. Some authors have reported on the reduction of virus local lesions (both number and size) and also on the reduction of infectivity per unit leaf area or fresh weight. Conversely, reports were also published showing that lesion number and infectivity could be stimulated by the administration of cytokinins to leaves.

Because we were the first who reported on the suppression of the virus in a local lesion host by kinetin (KIRÁLY and SZIRMAI, 1964), we wanted to have a deeper insight into the mechanism of reduction of lesion number and infectivity of tobacco mosaic virus (TMV) in Xanthi-nc tobacco. Our aim was to compare the reduction in lesion number in kinetin-treated half leaves with the reduction of virus infectivity of crude leaf extracts of the kinetin-treated halves as well as with the ultracentrifuged leaf extracts. Also, in untreated (control) half leaves the infectivity of TMV in the developed local lesions was compared to that of the kinetin-treated halves and the infective TMV-RNA was determined in the kinetin

treated half leaves in comparison with the control values. It seemed also indispensable to know more about the local lesion development on a cellular level in the kinetin-treated tissues and a possible systematization of the virus in the kinetin-treated leaves.

Materials and Methods

Virus and plants

Nicotiana tabacum L. cv. Xanthi-nc was grown under ordinary greenhouse conditions and used for experiments in the 10-leaf-stage. The Ul strain of TMV was cultured in *Nicotiana tabacum* L. cv. Samsun. Tobacco leaves infected with TMV were ground (1 g leaf per 10 ml tap water) with a pestle and mortar and the homogenate used to inoculate the leaves of experimental tobacco plants. No abrasive was added to the inoculum. At various intervals after kinetin treatment the whole leaf was inoculated with TMV by use of a glass rod.

Kinetin treatment

Kinetin (Calbiochem) at 20 μg/ml tap water was applied twice daily for 10–20 days by spraying the attached half leaves of Xanthi-nc tobacco plants. In each experiment we used 12 plants, and on each plant 6–8 leaves.

Virus infectivity

Virus infectivity in leaves of treated and untreated half leaves was deter mined by the local lesion assay. The infectivity of virus preparation was deter mined by inoculating leaves of *N. tabacum* cv. Xanthi-nc and counting the loca lesions produced. Samples, 5 g each, were taken from leaves which had been inoculated with TMV 48 h previously. Before extractions were made, the surface of the leaves was thoroughly washed with 2% NaOH and rinsed with running tap water for 5 min to eliminate possible surface contaminations. The samples for assay were ground with precooled mortar and pestle containing 12 ml 0.06 *M* phosphate buffer pH 7, and 0.05% 2-mercaptoethanol. Leaf homogenates were centrifuged at 5000 *g* for 30 min at 0°C and the supernatant was assayed on Xanthi-nc tobacco. These supernatants were further centrifuged at 105 000 *g* for 60 min at 4°C. The pellet was resuspended in 0.06 *M* phosphate buffer pH 7,0 or in the supernatants, and then assayed on Xanthi-nc.

Infective RNA

Infective RNA was extracted by the phenol method (FRAENKEL-CONRAT *et al.*, 1961). Five-five g samples were homogenized in 2 ml GPS buffer (0.1 *M* glycine, 0.05 *M* K_2HPO_4, 0.3 *M* NaCl, adjusted to pH 9.5) containing 1 ml of

10% bentonite and 5 ml water-saturated phenol. The homogenate was stirred for 45 min at 4°C. After centrifugation at 6000 *g* for 45 min at 0°C the water phase was extracted and an equal volume of phenol containing 1 ml of 10% bentonite was added. The RNA was precipitated by addition of two volumes of cold ethanol. After 2 h at −20°C the suspension was centrifuged at 6000 *g* for 30 min at 0°C. The supernatant was discarded and the precipitate was dissolved in 1 ml GPS buffer containing 0.5 ml 10% bentonite. To assay for infectivity two drops of the samples were dropped onto Xanthi-nc leaves and rubbed with a glass rod.

Staining with Evans blue

Experimental tobacco leaves were infiltrated with 1% aqueous solution of Evans blue (Fisher Scientific Company, St. Louis) at various intervals after virus inoculation. This pigment was infiltrated into the inoculated leaves with a hypodermic syringe, and, after a 15 min equilibration period, discs (12 mm in diameter) were examined by light microscopy (TURNER and NOVACKY, 1974).

Results

Infectivity of crude leaf extracts and the ultracentrifuged extracts in comparison with the reduced local lesion number

Half leaves of Xanthi-nc tobacco were sprayed with 20 ppm kinetin solution twice daily for 10−20 days before inoculation with TMV. This procedure proved to be successful in suppressing the number of local lesions on the kinetin-treated half leaves as compared to the water-sprayed control halves. In a series of experiments the extent of the reduction of local lesions was different and always dependent on the length of period of the application of kinetin. In each experiment infectivity of the kinetin-treated and water-treated half leaf extracts and that of the ultracentrifuged extracts was also determined. Table 1 shows the extent of reduction in lesion number as a result of kinetin treatment and the extent of reduction or stimulation in infectivity of the same kinetin-treated half leaf as well as the changes in infectivity after ultracentrifuging the extract of the same leaf material. As is seen, the suppression of local lesion number corresponds with changes in infectivity of the crude extracts and that of the ultracentrifuged material. However, the crude extract and particularly the ultracentrifuged extract of the kinetin-treated tissues were always more infective than was expected on the basis of the number of local lesions. It was also found that the infectivity of the ultracentrifuged extracts of the kinetin-treated tissues was about the same or higher than that of the water control when the reduction in lesion number was low.

We can not explain the increased infectivity of the ultracentrifuged extract of the kinetin-treated half leaves as compared to that of the crude extract. We

Table 1

Kinetin-induced changes in lesion number and in virus infectivity of TMV in Xanthi-nc tobacco

Experiments	Period of kinetin treatment in days	Lesion number in per cent of untreated half leaves*	Infectivity of crude extracts in per cent of untreated half leaves*	Infectivity of ultracentrifuged virus in per cent of untreated half leaves*
I.	18—20	28.9**	53.0	79.0
II.	14—17	44.2	70.8	86.8
III.	10—13	60.3	94.0	127.3

Each experiment consisted of 5 replications. In each replicate we used 12 plants and on each plant 6—8 leaves. The samples were assayed on 20 leaves of Xanthi-nc by the half leaf method.

* The correlation between lesion number and infectivity of the crude extract was highly significant ($P < 0.01$); between lesion number and infectivity of the ultracentrifuged extract the correlation was significant ($P < 0.05$).

** Mean number of lesions per half leaf for the water-sprayed control was 175.

tested for inhibitors in the supernatants of the ultracentrifuged extracts but did not find any additional inhibitory effect on virus infection when they were compared to water as control. More research is needed to find an explanation for this phenomenon.

It would seem, that the kinetin treatment actually increases the infectivity of virus in lesions which were able to develop. It seems logical to suppose that, as a result of kinetin treatment, more infective particles exist in local lesions or perhaps in the tissues between local lesions.

Table 2

Infectivity of TMV in lesions and in area between lesions after kinetin treatment

Experiments	Lesion number			
	Untreated		Kinetin-treated*	
	100 lesions	Area between lesions	100 lesions	Area between lesions
I.	111	0	145	0
II.	98	0	131	0
III.	163	0	199	0
IV.	165	0	248	0

Hundred-hundred samples were cut from the leaves with cork-borer (2 mm in diameter), ground in 3 ml buffer pH 6.9 and assayed on Xanthi-nc.

* Plants were treated with 20 ppm kinetin solution for 14 days.

Table 3

TMV-RNA infectivity in control and in kinetin-treated Xanthi-nc tobacco leaves

Experiments	Infectivity of TMV-RNA Lesion number	
	Untreated half leaves	Treated half leaves
I.*	45	47
II.*	63	67
III.*	52	52

In each experiment we treated 12 plants on each 6—8 leaves. The samples were assayed on 8—10 leaves of Xanthi-nc.

* The ratio of lesion numbers on control and kinetic-treated half leaves were 100 : 40 (I. experiment); 100 : 45 (II. experiment) and 100 : 46 (III. experiment), respectively.

It was experimentally determined that the infectivity of virus particles of 100 lesions from the kinetin-treated half leaves indeed exceeded infectivity of 100 lesions from the control halves (Table 2). Additionally, in evaluation of samples taken from tissues between local lesions, it was convincingly demonstrated that the virus did not become systemic as a result of the action of kinetin on suppressing the number of the visible lesions.

Table 3 shows the infective RNA content of the kinetin-treated half leaves in comparison with the water-treated control halves. In spite of the significant reduction in lesion number in the kinetin-treated half leaves, the infective RNA content did not change. One can suppose that the higher virus-RNA content of lesions of the kinetin-treated halves compensates for the reduction in lesion number.

Influence of kinetin treatment on the number of infection sites and on the suppression of necrotization

It is seen from the foregoing that more infective particles and more viral-RNA exist in local lesions as consequences of kinetin treatment, thus they compensate for the suppression of infection sites. This seems to be the situation on a macroscopic level, however, Figs 1 and 2 show that this is not the case on a microscopic level. We infiltrated 1.0% aqueous solution of Evans blue into the intercellular space of the leaves after inoculation at various time intervals, and searched for dead cells using a microscope. Evans blue selectively stains the necrotic cells in tissues (GAFF and OKONG'O-OGOLA, 1971). Surprisingly, many more necrotic single cells were seen on the kinetin-treated and virus-inoculated half leaves, than on the untreated but inoculated ones. In other words, the number of infection sites and the number of local lesions were not reduced by kinetin

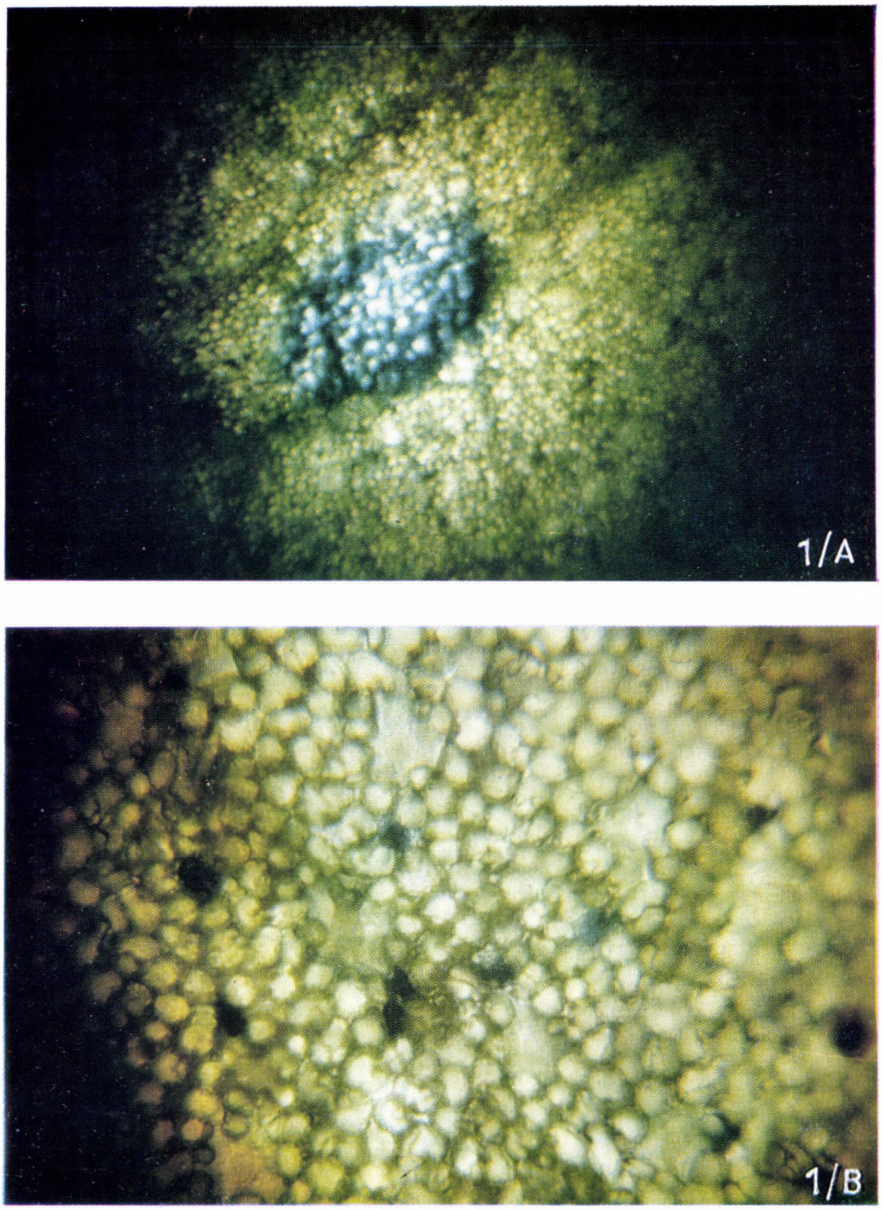

Fig. 1. Necroses induced by TMV in Xanthi-nc tobacco leaves (stained by Evans blue 48 h after inoculation). A: Visible local lesion on the control half leaf. B: Single-cell necroses on the kinetin-treated half leaf

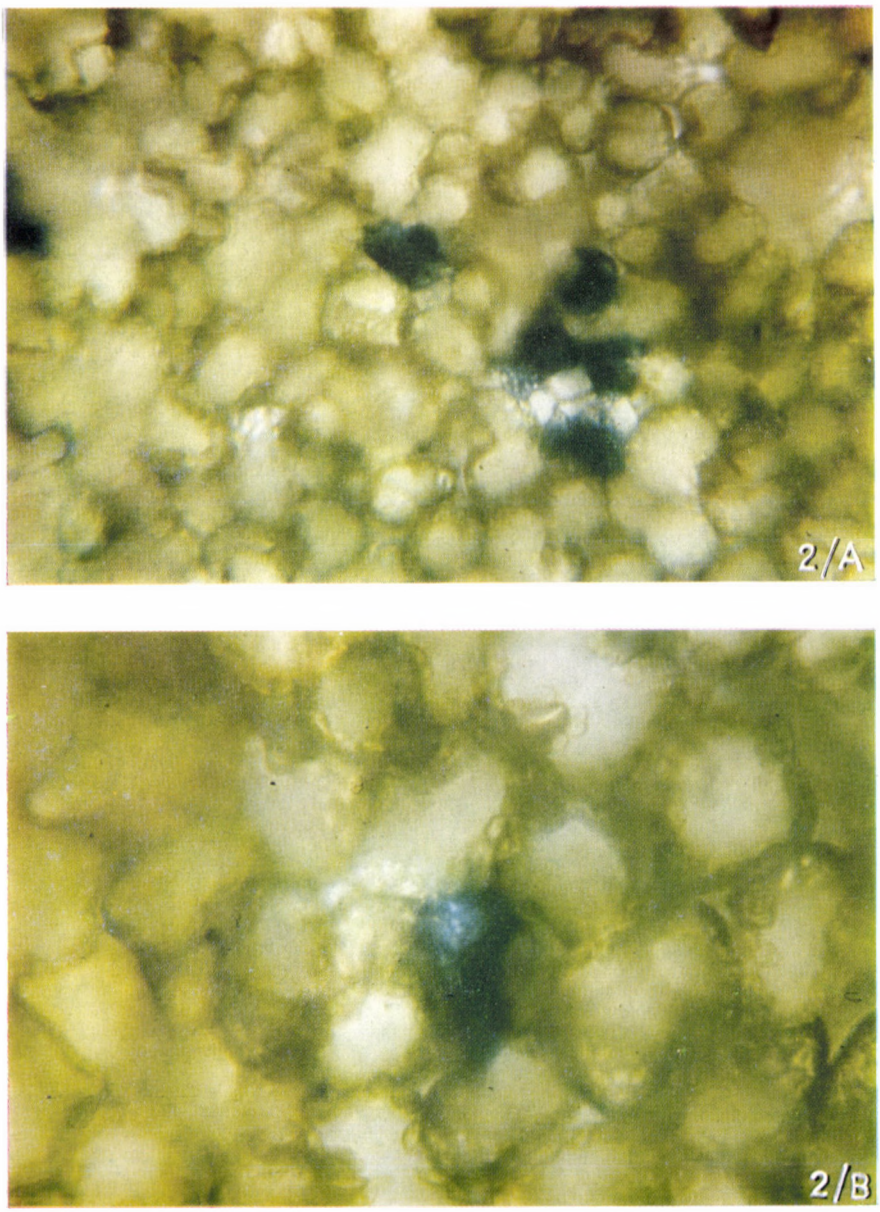

Fig. 2. Single-cell necroses on the kinetin-treated half leaf at different magnifications. A: A group of one-cell local lesions. B: A single-cell local lesion

treatment, only the size of the local lesions was influenced. The kinetin treatment suppresses only the number of necroses visible to the naked eyes, but the actual local lesion number is the same on the kinetin-treated and on the control half leaves.

Discussion

In a recent paper, ALDWINCLE (1975) called attention to the relation of timing of the first application of cytokinins to the inhibitory or stimulatory effect on virus infectivity. According to his theory, the application of cytokinins before inoculation may render host metabolism less susceptible to infection, e.g. virus take-over will slowing down under circumstances where the normal host RNA and protein syntheses are stimulated by cytokinins (cf. KIRÁLY *et al.*, 1968). However, after successful inoculation, the cell machinary is mostly used to produce viral RNA and viral protein, and then the cytokinin treatments might stimulate virus synthesis (cf. ALDWINCKLE and SELMAN, 1967).

This hypothesis, although in a modified form, seems to be applicable in our case where the treatment with kinetin took place only before virus inoculation. By reducing the number of visible lesions, kinetin seems to exert an inhibitory effect on TMV multiplication (in tissue area between visible lesions we were not able to show virus multiplication by bioassay). On the other hand, we found stimulation of virus infectivity in lesions of the kinetin-treated half leaves. Applying the speculation of ALDWINCKLE (1975) one can hypothesize that some cells in the tissue are very active in virus take-over and in this instance kinetin treatment will stimulate virus synthesis. This tissue area more or less corresponds to the areas of visible necrosis. In some other cells, the virus take-over will slowing down or even inhibited because the normal host RNA and protein syntheses are stimulated by kinetin. These areas would correspond to the one-cell necroses in the leaf. One can suppose that cells in leaf tissues are not uniformly susceptible to infection because the physiological state of the cells may be profoundly different. The non-uniform physiological state of cells in tissues have been demonstrated by several workers (cf. ATKINSON and MATTHEWS, 1967; MATTHEWS, 1970). Indeed ultrastructural and physiological differences of cells relate to a fine "mosaic" in the tissue (cf. WOOLHOUSE, 1967). It is clear to us that the above-mentioned speculation is a hypothesis with very few direct evidences. However, with the help of this hypothesis one can explain the controversial data on this field.

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Antagonistic Effect on TMV Infectivity Between Poly-L-lysine and Poly-L-arginine

By

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Polylysine pretreatment of Xanthi-nc tobacco half leaves caused a reduction of TMV local lesions compared to the control. A similar polyarginine pretreatment resulted in an increase in local lesions. A mixture of the polycations used for pretreatment did not cause reduction or increase of the number of local lesions. Using a systemic host we demonstrated the same antagonistic effect of these two polycations.

On the other hand we got similar results if we incubated the pure virus with polylysine or polyarginine. Therefore, these synthetic polycations could affected the replication of TMV in some way, perhaps through the pinocytosis mechanism, or membrane alterations are enhanced by basic polypeptides.

Reports from some laboratories have been shown that several polycations (e.g. polylysine and polyornithine) inhibit tobacco mosaic virus infectivity (STAHMANN *et al.*, 1951; STAHMANN and GOTHOSKAR, 1958; ZHURAVLEV *et al.*, 1974). STEIN and LOEBENSTEIN (1972) reported that synthetic polyanions would induce interference with the infection of tobacco mosaic virus. However, SHAW (1972) using poly-L-ornithine, has shown that this polycation produced an increase in the amount of TMV retained by inoculated tobacco leaves and in the amount of uncoating of the RNA of this retained virus. The results of these experiments with polyornithine do not agree with the conclusions from work a similar substance, polylysine. STAHMANN et al. (1951) found that mixtures of polylysine and TMV produced fewer lesions on a local lesion host than did TMV alone. The effect was attributed to the formation of ionic bonds between the ϵ-NH_2 groups of polylysine and the acidic group of the virus (BURGER and STAHMANN, 1951). These studies led to the conclusion that polybasic substances inhibit TMV by combining the virus and not by an effect on the host and that this combination prevents either the initial binding of the virus by the host cell or some other cell-virus interaction (STAHMANN and GOTHOSKAR, 1958). SHAW's (1972) experiments have shown that polyornithine treatment appeared to increase rather than inhibit the initial interaction of virus and host and, further to provide conditions which stimulates the release of the RNA of the virus. Polyornithine greatly increases for the infection of the tobacco protoplasts by TMV (TAKEBE and OTSUKI, 1969).

These contradictory data suggest that the decreased or increased infectivity

could not be attributed simply to a polyanion (virus)–polycation (polybase) complex formation. Also here we must note that there are several reports that basic polypeptides (of different molecular weights) are on or in several viruses, and this may have a role in the virus–host interaction and virus replication (GINOZA *et al.*, 1954; HERSHEY, 1955; FINE *et al.*, 1968; RUSSEL *et al.*, 1968; FLEISSNER, 1971; GIBSON and ROIZMAN, 1971; COURTNES and BENYESH-MELNICK, 1974; KRELL and LEE, 1974; BALÁZS *et al.*, 1975; POGO *et al.*, 1975). Therefore we performed experiments with polylysine and polyarginine to determine their effects on virus infectivity and virus–cell interaction.

Materials and Methods

The U_1 strain of TMV was cultured in *Nicotiana tabacum* L. cv. Samsun plants. The virus was purified according to FRAENKEL-CONRAT (1966) and GOODING and HEBERT (1967). To determine the effects of polycation induced virus susceptibility, we used *Nicotiana tabacum* L. cv. Xanthi-nc (resistant-host) and *Nicotiana tabacum* L. cv. Xanthi (susceptible-host). All tobacco plants were grown under normal greenhouse conditions and were used for the experiments at the 6 to 8 leaf stage.

Poly-L-arginine-HCl (Sigma Chemical Co. St. Louis, Mo.) type 11-B. Mol. wt. 17,000 and the degree of polymerization 81, and the polyornithine content was 3–5%. Poly-L-lysine-HCl (Sigma Chemical Co. St. Louis, Mo.) type V-A. Mol. wt. 30 000.

Polycations were applied at 10 μg/ml in 0.1 *M* phosphate buffer pH 6.9 by hypodermic syringe infiltration of one half of attached leaves. The control half leaves were infiltrated with 0.1 *M* phosphate buffer pH 6.9 containing 10 μg/ml L-lysine-HCl (Calbiochem La Jolla, Cal.) or 10 μg/ml L-arginine-HCl (BDH Poole, England) while in some experiments we used buffer infiltrated half leaves as control.

The purified virus at 100 μg/ml in 0.1 *M* phosphate buffer pH 6.9 was mixed with each the above mentioned polycations separately or with the amino acids so that the purified virus solution contained 10 μg/ml from each polycation or each amino acid. The solution after mixing was incubated for 10 min and used for inoculation of half leaves (0.1 ml/half leaf). No abrasive was added to the inoculum and leaves were washed with tap water after inoculation (cf. TAKEBE and OTSUKI, 1969).

A Latin-square arrangement of inoculated leaves minimized variation in number of lesions due to individual plant and leaf variability. Local lesions were counted 2–4 days after inoculation, depending on greenhouse conditions.

Virus multiplication in leaves of Xanthi tobacco was determined by local lesion assay. Six leaf disks (about 600 mg) were used for inoculation of *Nicotiana tabacum* L. cv. Xanthi-nc, and local lesions were counted.

Results and Discussion

Xanthi-nc tobacco half leaves that were infiltrated with polylysine (10 μg/ml) and after the solution evaporated were inoculated with TMV, produced

Table 1

Effect of polylysine and polyarginine on the susceptibility of Xanthi-nc tobacco leaves to TMV

Treatment	Lesion number on the half leaves
Control (buffer with lysine, 10 μg/ml)	1023
Polylysine (10 μg/ml in buffer)	767
Control (buffer with arginine, 10 μg/ml)	1240
Polyarginine (10 μg/ml in buffer)	1771
Polylysine (10 μg/ml in buffer)	880
Polyarginine (10 μg/ml in buffer)	1560

Values represents the mean of five replications. One half of the leaf was injected with polycations and the opposite half with buffer contained one of amino acid as control. The whole leaf was then inoculated after evaporation with TMV using a glass rod. Control (buffer alone) = 1150.

fewer lesions compared to the buffer or 10 μg/ml lysine in buffer infiltrated halves (Table 1). These data confirm the works of STAHMANN (1951), and of BURGER and STAHMANN (1951). However infiltration of 10 μg/ml polyarginine into the half leaves of Xanthi-nc tobacco leaves and then inoculation with TMV caused many more lesions than on the buffer treated control, or on the 10 μg/ml arginine infiltrated halves (Table 1). If we mixed the two polycations they antagonized each other.

Half leaves of the systemic host (Xanthi) infiltrated with polylysine produced fewer virus particles compared to the control half leaves, and polyarginine in the same host induced greater virus replication (Table 2). The relative virus concentration was tested on hypersensitive tobacco Xanthi-nc.

When we inoculated the tobacco leaves with a mixture of pure tobacco mosaic virus and polylysine or a mixture of pure TMV and polyarginine we got lower infectivity of TMV mixed with polylysine, and higher infectivity of TMV mixed with polyarginine (Table 3). If we mixed together the two above mentioned

Table 2

Effect of polylysine and polyarginine on the multiplication of TMV in Xanthi tobacco. Values calculated from relative virus concentration of TMV-inoculated half leaves tested on hypersensitive Xanthi-nc

Treatment	Lesion number 2 days after inoculation	Lesion number 3 days after inoculation
Control (buffer alone)	680	1358
Control (buffer with lysine 10 μg/ml)	696	1312
Polylysine (10 μg/ml in buffer)	528	920
Control (buffer with arginine 10 μg/ml)	694	1322
Polyarginine (10 μg/ml in buffer)	822	1765
Polylysine (10 μg/ml in buffer)	560	950
Polyarginine (10 μg/ml in buffer)	828	1854

Xanthi half leaves were injected with 0.1 M phosphate buffer (pH 6.9) or polycation solution in the same buffer and after evaporation were inoculated with TMV. Six leaf disks (18 mm in diameter) were extracted in 3 ml phosphate buffer at 48 h and 72 h after inoculation. Then 10 half leaves of Xanthi-nc were inoculated with the diluted extracts by using a glass rod. No abrasive was added to the inoculum. Values represent means of three replications.

polycations with the virus solutions we could not detect any changes in virus infectivity. These data were established in both host-virus systems, susceptible and resistant. The antagonism between polylysine and polyarginine may suggest a partial explanation of this effect.

Tobacco leaves retain large amounts of basic proteins, but almost none of a highly acidic protein when they are applied in the manner used for virus inoculations (SHAW, 1972). This suggests that large areas of the surface layers of tobacco are negatively charged. The TMV particles may require positively charged receptor sites for their attachment to the leaf surface. Therefore it is possible that similar to the polyornithine effect (SHAW, 1972), the effect of polyarginine on the attachment of TMV may be the result of a decrease in the electronegativity of this leaf area and the availability of a larger number of positively charged virus particles. However these ideas and results do not agree with STAHMANN's results with polylysine or with our polylysine results. The effects of polyornithine and polyarginine could also result from stimulation of a mechanism whereby virus particles either attach themselves to, or penetrate cell membranes. Pinocytosis, phagocytosis and

Table 3

Effect of polylysine and polyarginine on the TMV infectivity

Test plant — Polycation	Local lesions 10 half leaves		% Change due to polycation
	virus alone	virus plus polycation	
Xanthi-nc — polylysine	1118	552	−51%
(A) polyarginine	1200	1548	+29%
polylysine + polyarginine	1190	1206	>+ 1%
Xanthi — polylysine	1095	603	−45%
(B) polyarginine	1140	1640	+44%
polylysine + polyarginine	1121	1100	>− 1%

All inocula contained 100 μg/ml TMV in 0.1 M phosphate buffer pH 6.9. All polycations were used at 10 μg/ml.

Relative infectivities of TMV alone and TMV mixed with polycations are indicated by numbers of lesions on inoculated half-leaves of Xanthi-nc (A).

Relative infectivities of TMV alone and TMV mixed with polycations were also tested on Xanthi tobacco leaves. Data in table originated from six leaf disks extracted in 3 ml phosphate buffer and assayed on Xanthi-nc (B).

Absolute control of A—1169. Absolute control of B—1115.

other membrane alterations in many cells are enhanced by basic polypeptides (KATCHALSKI *et al.*, 1964). These mechanisms have been suggested as mechanisms by which viruses enter and infect plant cells (COCKING and POYNAR, 1969). It is noteworthy that several viruses have been shown to be associated with basic proteins (POGO *et al.*, 1975; BALÁZS *et al.*, 1975; FINE *et al.*, 1968; FLEISSNER, 1971; GIBSON and ROIZMAN, 1971; HERSHEY, 1955; GINOZA *et al.*, 1954).

These phenomenon suggest that these basic proteins or polypeptides have a role in virus attachment, virus-RNA uncoating and in the replication.

Our results suggest that the two type of polycations could affect the virus attachment and or the virus replication.

Probably this phenomenon depends on the specificity of the interaction of nucleotides with basic polypeptides. RIFKIND and EICHHORN (1970) established that the helical conformation of polyarginine stabilized more readily than that of polylysine. It is also possible, that the antagonistic effect could originate from the different structure of the two basic amino acids, i.e. reaction differences between ϵ-NH_2 and guanidino group (different protonation).

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Natural Occurrence of Celery Mosaic Virus in Hungary

By

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A virus isolated from mosaic diseased parsley (*Petroselinum crispum* [Mill.] Nym. cv. *Szigetközi*), celery (*Apium graveolens* L. convar. *dulce* [Mill.] Rothm.), carrot (*Daucus carota* L.) and parsnip plants (*Pastinaca sativa* L.) from Mosonmagyaróvár, Vonyarcvashegy, Balatonszentgyörgy and Keszthely in Hungary, was identified as celery mosaic virus (CeMV, ∗/∗ : ∗/∗ : E/E : S/Ap). The four isolates of CeMV (Pc, Ag, Dc and P) were transmitted by mechanical inoculation and also by *Myzus persicae* Sulz aphids in a stylet-borne manner. By mechanical inoculation ten species in three families became infected out of 29 species in 9 families tested. The infected plants were *Ammi majus* L., *A. visnaga* (L.) Lam., *Apium graveolens* L., *Chenopodium amaranticolor* Coste et Reyn., *Ch. murale* L., *Ch. quinoa* Willd., *Cicuta virosa* L., *Coriandrum sativum* L., *Daucus carota* L. and *Petroselinum crispum* (Mill.) Nym. *Ammi visnaga* and *Cicuta virosa* were new experimental hosts of the four isolates of CeMV. The Pc, Ag, Dc and P-isolates reacted positively to antisera of an 20 N strain of CeMV. In electron microscopy using dip method, long flexuous filaments, about 760—770 nm in length were observed. In the course of cytological examinations cytoplasmic inclusion bodies were found. In the electron microscope investigation of ultrathin sections of infected *Ammi majus* plant tissue revealed numerous lamellar aggregates in the cytoplasm. It was also observed pinwheel structures which are characteristic for CeMV (potyvirus group). For the four isolates of CeMV the following physical properties were established: thermal inactivation point 54—56°C (Ag, Dc and Pc-isolates), 56—58°C (P-isolate), dilution end point 10^{-2}—2×10^{-3} (Ag, Dc, Pc-isolates), 10^{-3}—2×10^{-4} (P-isolate), longevity in vitro 7 days.

This is the first record of CeMV in Hungary.

On the basis of results of more than half a century of research work concerning the virus infection of economically more or less important vegetable-, fodder-, medicinal-, condiment-, ornamental- and weed plants belonging to the family *Umbelliferae (Apiaceae)*, virus diseases of some forty plant species can be said to be known at present (reviewed by WOLF and SCHMELZER, 1972). Of the fifteen viruses isolated so far from various plant species the western celery mosaic virus (syn.: celery mosaic virus, CeMV; ∗/∗ : ∗/∗ : E/E : S/Ap) described by SEVERIN and FREITAG (1938) in California is considered to be most frequent. It is an about 770—780 nm long, flexuous virus with filamentous particle, transmissible both mechanically and by numerous aphid species in a stylet-borne manner (KARL and WOLF, 1974). This virus is a member of the potyvirus group and it has a narrow range of hosts. Considering that plant virological investigations in Hun-

gary have paid so far very little attention to plants belonging to the family *Umbelliferae*, the results of an extensive research work carried on in Europe, first of all in England and Germany (WATSON, 1960; WATSON and SERJEANT, 1964; WATSON *et al.*, 1964; HOLLINGS, 1964; 1965; BRANDES and LUISONI, 1966; MURANT and GOLD, 1968; HEINZE, 1968; WOLF, 1968; MURANT *et al.*, 1969; WOLF, 1969; WOLF *et al.*, 1969; WOLF, 1970; WALKEY *et al.*, 1970; SUTABUTRA and CAMPBELL, 1971; WALKEY and COOPER, 1971a, b; WOLF, 1972; WOLF and SCHMELZER, 1973; PEMBERTON and FROST, 1974; BUTURAC, 1974; BUTURAC *et al.*, 1974; KEMP and FROWD, 1975) encouraged us to start etiological studies on symptoms characteristic of virus diseases observed earlier in Hungary in the following plants: *Pastinaca sativa* L., *Apium graveolens* L. convar. *dulce* (Mill.) Rothm., *Petroselinum crispum* (Mill.) Nym. cv. *Szigetközi* and *Daucus carota* L. convar. *sativus* (Hoffm.) Arcang.

Material and Methods

Naturally infected plants of parsley (*Petroselinum crispum*), celery (*Apium graveolens*), carrot (*Daucus carota*) and parsnip (*Pastinaca sativa*) were collected in private gardens at Keszthely, Vonyarcvashegy and Balatonszentgyörgy (on the shores of Lake Balaton, Hungary) and in the Horticultural Experiment Station of the Faculty of Agricultural Sciences, Mosonmagyaróvár. All collected plants showed very severe vein clearing, ochre mosaic spots; in addition to these symptoms the parsnip plants showed highly remarkable leaf crinkling (Fig. 1A and Fig. 2).

The virus was mechanically transmitted from diseased parsley-, celery-, carrot- and parsnip plants separately by homogenizing the infected leaf tissue (1 : 1 w/v) in 0.1 *M* phosphate buffer of pH 7.0, and rubbing the leaves in usual manner (e.g. *Ammi majus* L., *A. visnaga* [L.] Lam., *Capsicum annuum* L., *Chenopodium amaranticolor* Coste et Reyn., *Ch. murale* L., *Ch. quinoa* Willd., *Coriadrum sativum* L., *Cucumis sativus* L., *Datura stramonium* L., *Gomphrena globosa* L., *Nicotiana clevelandii* A. Gray, *N. glutinosa* L., *N. tabacum* L. *Bel 61—10*, *Samsun* and *Xanthi-nc*, as well as *Phaseolus vulgaris* L. cv. *Red Kidney* and *Tetragonia tetragonoides* [Pall.] O. Ktze, etc.).

For aphid transmission studies *Myzus persicae* Sulz. aphids were reared on *Brassica rapa* L. var. *rapa* plants were fasted for three hours, then brush-transferred to the virus donor *Ammi majus* plants for an aquisition feeding period of 5—8 minutes. Ten aphids were then transferred to healthy *Ammi majus* and *A. visnaga* plants and killed afterwards with Phosdrin insecticide.

Serological reactions were studies using an antiserum (titre 1 : 2048 in slide precipitin test) against the normal strain (20 N) of CeMV infecting only umbelliferous plants. The antiserum was kindly supplied by Prof. Dr. E. LOVISOLO and Prof. Dr. E. LUISONI (Torino, Italy). For electron microscopy the preparations were taken from infected *Ammi majus* leaves. Leaf dip were negatively stained

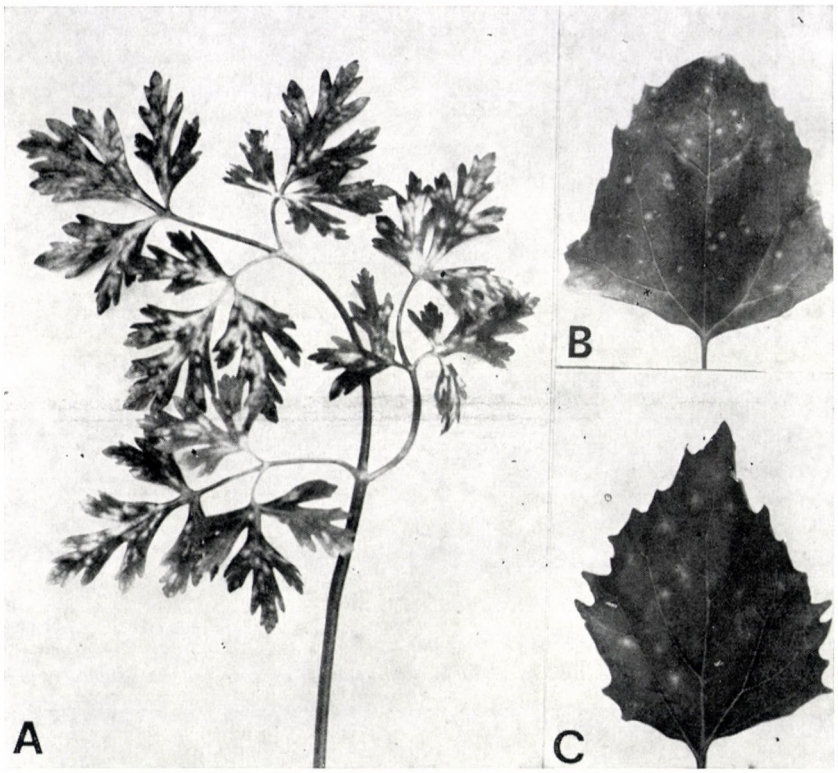

Fig. 1. Field symptoms of celery mosaic virus on *Petroselinum crispum* (Mill.) Nym. cv. *Szigetközi* (A) and local lesions on the leaves of *Chenopodium amaranticolor* Coste et Reyn. (B) and *Ch. quinoa* Willd. (C) test plants artificially inoculated with the Pc-isolate of celery mosaic virus from *Petroselinum crispum* (Mill.) Nym. cv. *Szigetközi*

on carbon coated grids with 4 per cent potassium phosphotungstate, pH 7.2. The examination of virus particles was carried out with a Siemens Elmiskop I. type electron microscope.

The light microscopical investigations of inclusion bodies involved exclusively living cells. Both inoculated and healthy *Ammi majus* plants were studied microscopically. For electron microscopical investigations strips of leaf tissue from infected *Ammi majus* plants were fixed for 30 min in 1 per cent (v/v) glutaraldehyde in cacodylate buffer pH 7.2 and postfixed for 2 hr in 1 per cent (w/v) osmium tetroxide. After fixation, samples of tissue were dehydrated in ethanol series and embedded in Araldite (see JURETIĆ, 1974). Sections were cut with Reichert OM U2 ultramicrotome and examined in Siemens Elmiskop I. Sap from systemically infected *Ammi majus* leaf tissue ground in distilled water (1 : 1 v/v) was used to determine the physical properties of the CeMV.

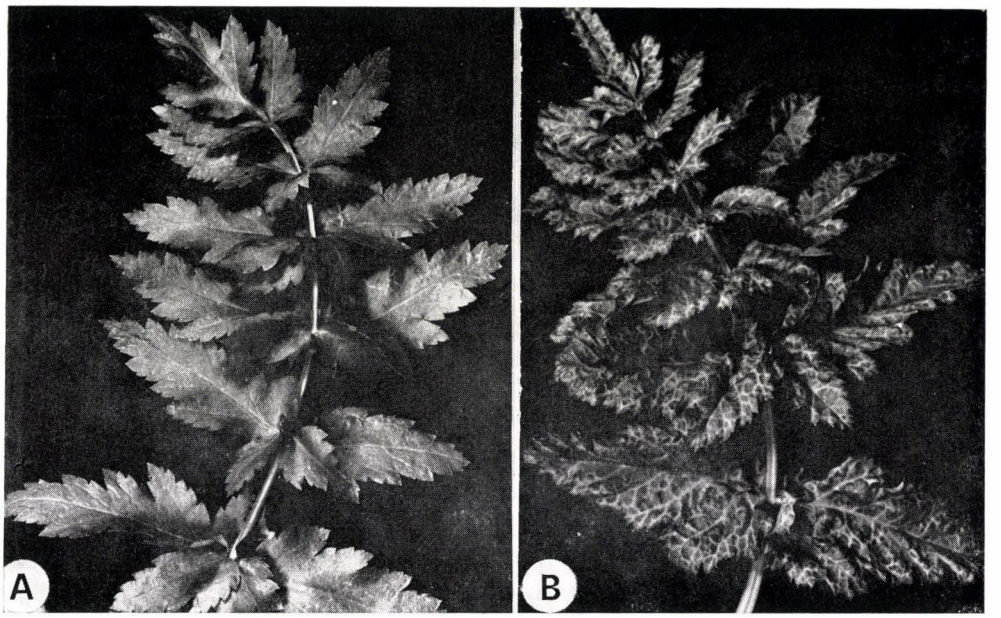

Fig. 2. Symptoms produced by celery mosaic virus (P-isolate) on *Pastinaca sativa* L. (B) and healthy leaf (A) of *Pastinaca sativa* L.

Results and Discussion

In the course of studying the host range of the virus isolated from parsley-, celery-, carrot- and parsnip plants 29 plant species and varieties, respectively, belonging to nine plant families (*Aizoaceae*, *Amaranthaceae*, *Basellaceae*, *Chenopodiaceae*, *Cucurbitaceae*, *Fabaceae [Leguminosae, Papilionaceae]*, *Malvaceae*, *Solanaceae*, *Umbellifereae [Apiaceae]*) were artificially inoculated. Three species belonging to the family *Chenopodiaceae* (*Chenopodium amaranticolor*, *Ch. murale*, *Ch. quinoa*) responded with chlorotic local lesions to the inoculation. The lesions sometimes became necrotic in the centre, or occasionally developed a red margin on *Ch. amaranticolor* (Fig. 1B and C). Of the seven susceptible plant species belonging to the family *Umbelliferae (Apiaceae)* — *Apium graveolens*, *Cicuta virosa*, *Coriandrum sativum*, *Daucus carota*, and *Petroselinum crispum* — reacted with systemic vein clearing and mosaic symptoms; *Ammi majus* and *A. visnaga* showed local and systemic chlorotic spots, filiform leaves and a premature necrotic disintegration of the inflorescence (Fig. 3). In the course of our investigations *Ammi visnaga* and *Cicuta virosa* proved to be new experimental hosts. Other hosts commonly used as virus indicators were tested to, but none of the isolates infected any of the following plants: *Basella rubra* L., *Capsicum annuum*, *Cucumis sativus*,

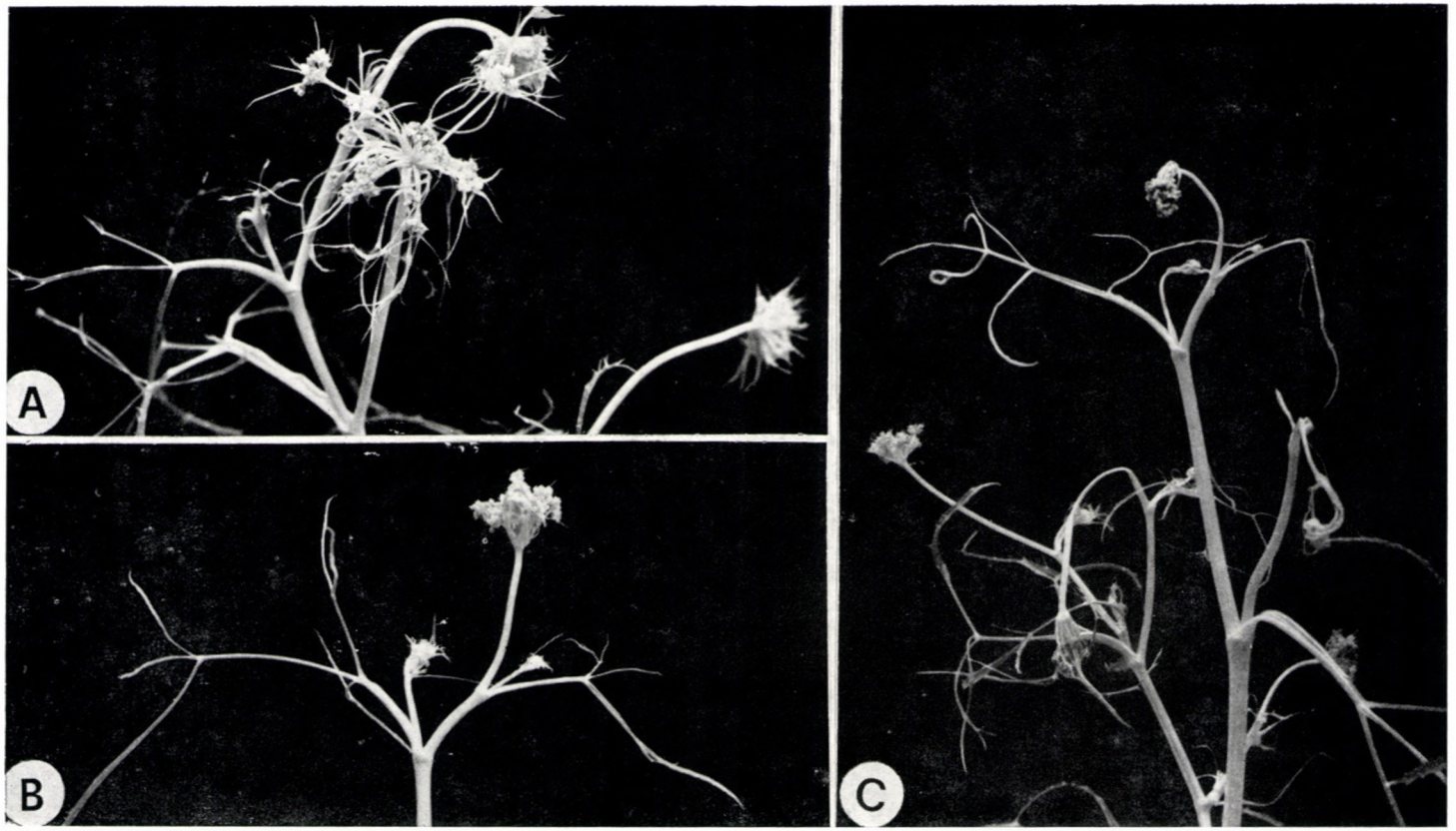

Fig. 3. *Ammi majus* L. plants inoculated in the laboratory with P-isolate of celery mosaic virus. A: 30 days p. i., B: 35 days p. i., C: 45 days p. i.

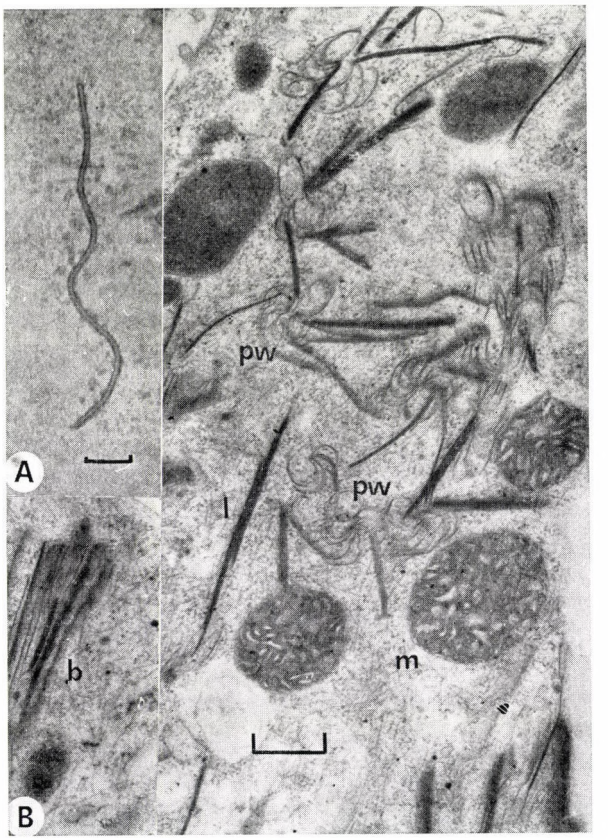

Fig. 4. Long flexuous filaments of the P-isolate of celery mosaic virus (A). Approx. × 50 000, bar represents 100 nm. Electron micrograph of section of infected *Ammi majus* L. plant, showing pinwheel structures (B). pw: pinwheel structures in cross-section, b: pinwheel structures in longitudinal section, l: lamellar aggregates in the cytoplasm, m: mitochondria. Approx. × 30 000, bar represents 500 nm

Datura stramonium, *Gomphrena decumbens* Jacq., *G. globosa*, *Malva verticillata* L., *Nicotiana clevelandii*, *N. glutinosa*, *N. tabacum* cv. *Bel 61 – 10*, *Samsun*, *Xanthi-nc*, *Obione sibirica* (L.) Fisch., *Petunia atkinsiana* Don., *P. hybrida* Vilm., *Phaseolus vulgaris* cv. *Red Kidney*, *Tetragonia crystallina* L'Hérit., *T. echinata* Ait., *T. tetragonoides*. We should like to point out here that *Nicotiana clevelandii* found susceptible to CeMV by IWAKI and KOMURO (1970) did not prove to be susceptible to the four CeMV isolates examined. The virus was not recovered from inoculated plants to *Chenopodium amaranticolor*.

By *Myzus persicae* aphids the isolates were readily transmitted in stylet-borne manner from previously virus-inoculated to healthy young *Ammi majus*

and *A. visnaga* plants. In two trials *Myzus persicae* transmitted all four isolates, infecting a total of 17 out of 20 plants.

In the course of investigations made with CeMV antiserum we found the isolates Ag (*Apium graveolens*), Dc (*Daucus carota*), Pc (*Petroselinum crispum*) and P (*Pastinaca sativa*) to be identical with CeMV. Considering that in addition to, and together with CeMV the above plants most frequently have been infected with cucumber mosaic virus (CMV, cf. WOLF, 1970; WOLF and SCHMELZER, 1972; 1973), we carried out serological examinations with CMV-antiserum in agar gel double diffusion test. As expected on the basis of the results of host range investigations, the presence of CMV could be pointed out in none of the isolates.

During the electron microscope studies we found 760–770 nm long flexuous filaments characteristic of CeMV belonging to the potyvirus group (Fig. 4A).

In the course of cytological examinations cytoplasmic inclusion bodies characteristic of the potyvirus group were found. They showed a fine granular structure. Inclusion bodies could not be seen in healthy *Ammi majus* plants. Our electron microscope investigation of ultrathin sections of tissue revealed numerous lamellar aggregates in the cytoplasm. It was also observed pinwheel structures which are characteristic for the potyvirus group (Fig. 4B). Pinwheel structures were found in tissue infected with CeMV in papers by PURCIFULL and SHEPARD (1967), BUTURAC (1974), BUTURAC *et al.* (1974).

In studying the physical properties we found that the thermal inactivation point of isolates Ag, Dc and Pc was 54–56°C, their dilution end point 10^{-2}–2×10^{-3} and longevity *in vitro* maximum 4 days. Isolate P had thermal inactivation point at 56–58°C, dilution end point at 10^{-3}–2×10^{-4}, while its longevity *in vitro* was somewhat more than that for the other isolates, a total of 7 days.

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Circaea lutetiana L. (Family: *Onagraceae [Oenotheraceae]*), a New Natural Host of Cucumber Mosaic Virus

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Cucumber mosaic virus (R/1 : 1/18 : S/S : S/Ap) was isolated from naturally infected *Circaea lutetiana* L. plants in Hungary. On the basis of host range, aphid transmissibility, serology, plant protection test and physical properties the virus was identified as a green strain of cucumber mosaic virus (designated CMV-G/Cir). Thirteen species are recorded as experimental hosts for CMV-G/Cir. The virus showed stylet-borne transmission by *Myzus persicae* Sulz., and reacted in gel-diffusion test with CMV antiserum. The plant protection test between CMV-G/Cir and the white (W) strain of cucumber mosaic virus (CMV-W) resulted in protection of *Nicotiana tabacum* L. cv. *Xanthi-nc* against the multiplication of CMV-W in challenging inoculations. This is the first report of CMV-G/Cir occurring in the highly important perennial virus reservoir *Circaea lutetiana.*

According to our present knowledge the number of plant species in the family *Onagraceae (Oenotheraceae)* showing susceptibility to various viruses is relatively low. A review of earlier literature reveals that so far some 19 plant species have been examined for susceptibility to 22 viruses and two mycoplasmas. According to the results of investigations 10 of the examined species proved susceptible to 13 viruses and two mycoplasmas, while 14 species were found to be resistant to 15 viruses (for references see Table 1).

In the course of virological and botanical collecting tours made in the last several years our attention was caught by a virus disease of *Circaea lutetiana* L., a perennial plant widely occurring in the humid, rocky forests, groves and fen-woods of Hungary. Considering that — to our best knowledge — no data on the spontaneous virus susceptibility of *Circaea lutetiana* are available in the world literature, we carried out investigations to identify the virus pathogen causing the disease.

Table 1

Onagraceae (Oenotheraceae) plants susceptible and/or insusceptible to several plant viruses and mycoplasmas

Plants	Viruses	Reaction*	Literature
Circaea lutetiana L.	Tobacco mosaic virus	−	HOLMES (1938)
Clarkia sp.	Alfalfa mosaic virus	+	QUANTZ (1956)
C. elegans Gougl.	Alfalfa mosaic virus	−	PRICE (1940)
	Aster yellows mycoplasma	+	SEVERIN and FRAZIER (1945)
	Beet curly top virus	+	FREITAG and SEVERIN (1936)
	Cucumber mosaic virus	+	PRICE (1940)
	Tobacco necrosis virus	+	PRICE (1940)
	Tobacco etch virus	−	HOLMES (1946)
	Tomato ring spot virus	+	PRICE (1940)
	Tobacco ring spot virus	+	PRICE (1940)
	Radish mosaic virus	−	TOMPKINS (1939)
	Turnip mosaic virus	−	TOMPKINS *et al.* (1938)
C. pulchella Pursh.	Alfalfa mosaic virus	+	SCHMELZER *et al.* (1973)
	Tobacco rattle virus	+	SCHMELZER (1957)
Epilobium angustifolium L.	Beet western yellows virus	+	WALLIS (1967)
	Beet yellows virus	−	ROLAND and TAHON (1961)
E. californicum Hausskn.	Aster yellows mycoplasma	+	FRAZIER and SEVERIN (1945)
	Alfalfa dwarf mycoplasma	+	FRAZIER and SEVERIN (1945)
E. montanum L.	Anemone mosaic virus	−	HOLLINGS (1957a)
	Chrysanthemum mild mosaic virus	−	HOLLINGS (1957b)
E. paniculatum Nutt.	Alfalfa dwarf mycoplasma	+	FREITAG (1951)
	Aster yellows mycoplasma	+	FRAZIER and SEVERIN (1945)
	Beet yellows virus	−	ROLAND and TAHON (1961)
Fuchsia magellanica Lam.	Alfalfa dwarf mycoplasma	+	FREITAG (1951)
	Tomato aspermy virus	−	BRIERLEY *et al.* (1955)
F. speciosa hort.	Beet mosaic virus	−	SCHMELZER (1959)
	Tobacco rattle virus	−	SCHMELZER (1957)
Godetia amoena G. Don.	Cauliflower mosaic virus	−	WALKER *et al.* (1945)
	Cucumber mosaic virus	+	PRICE (1940)
	Alfalfa mosaic virus	+	PRICE (1940)
	Tobacco necrosis virus	+	PRICE (1940)
	Tobacco ring spot virus	+	PRICE (1940)

Plants	Viruses	Reaction*	Literature
	Tomato ring spot virus	+	PRICE (1940)
	Turnip mosaic virus	+	WALKER *et al.* (1945)
G. grandiflora Lindl.	Aster yellows mycoplasma	+	SEVERIN and FREITAG (1934)
	Alfalfa dwarf mycoplasma	+	FREITAG (1951)
	Beet mosaic virus	−	SCHMELZER (1959)
	Tobacco rattle virus	−	SCHMELZER (1957)
	Tomato spotted wilt virus	+	GARDNER *et al.* (1935)
	Turnip mosaic virus	−	TOMPKINS *et al.* (1938)
	Radish mosaic virus	−	TOMPKINS (1939)
Oenothera biennis L.	Beet western yellows virus	+	WALLIS (1967)
	Tobacco streak virus	+	FULTON (1948)
O. clutei A. Nelson	Nasturtium ring spot virus**	−	SCHMELZER (1960)
	Tobacco rattle virus	−	SCHMELZER (1957)
	Beet mosaic virus	−	SCHMELZER (1959)
O. fruticosa L.	Beet mosaic virus	−	SCHMELZER (1959)
	Tobacco rattle virus	−	SCHMELZER (1957)
O. hookeri Torr. et Gray.	Alfalfa dwarf mycoplasma	+	FREITAG (1951)
O. lamarckiana hort.	Tobacco etch virus	−	HOLMES (1946)
	Tobacco mosaic virus	−	HOLMES (1946)
O. missouriensis Sims.	Pea mosaic virus	−	MURPHY and PIERCE (1937)
O. speciosa Nutt.	Beet mosaic virus	−	SCHMELZER (1959)
	Tobacco rattle virus	+	SCHMELZER (1957)

* Susceptible: +, non susceptible: −
** Syn.: Broad bean wilt virus

Material and Methods

On the leaves of *Circaea lutetiana* plants showing a mass occurrence in the fenwoods surrounding in Keszthely and Héviz typical mosaic spots were found. The diseased plants showed marked growth inhibition and the leaves were highly deformed (Fig. 1). In the course of the experiments plant sap was extracted from those leaves in 0.1 *M* phosphate buffer, pH 7.0, and test plants (see Table 2) were inoculated using carborundum (mesh 500) as abrasive.

Aphid transmission of the virus from *Circaea lutetiana* (designated by Cir-isolate) was studied with apterous *Myzus persicae* aphids starved for 3–4 hours. Naturally infected *Circaea lutetiana*, further *Nicotiana tabacum* cv. *Xanthi-nc* plants, young *Cucumis sativus* L. and *N. tabacum* cv. *Xanthi-nc* plants were used as test plants. Each test plants was inhabited with 10 aphids. The aphids were destroyed with the insecticide Phosdrin at the end of test feeding.

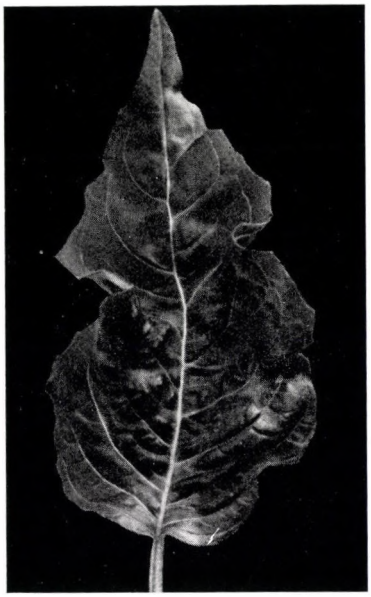

Fig. 1. Leaf of *Circaea lutetiana* L. spontaneously infected with cucumber mosaic virus

The serological tests were performed by the method of double diffusion in 0.9 per cent agar gel containing standard additives. Crude extracts of infected and healthy leaves of *Cucumis sativus* and *Nicotiana glutinosa* were used in the experiments. Antiserum against CMV (supplied by Prof. Dr. Y. KOMURO) was at our disposal for serological tests. Homologous antiserum titer was 1 : 256.

In the course of plant protection tests young *Nicotiana tabacum* cv. *Xanthi-nc* plants were first inoculated with the Cir-isolate derived from diseased *Circaea* plants. After the systemic symptoms had appeared the *Xanthi-nc* tobacco plant were inoculated with the W-strain of CMV as challenge strain.

Physical properties of the Cir-isolate were examined using extracted infectious *Cucurbita pepo* L. convar. *patissonina* Greb. f. *radiata* Nois. sap, with *Chenopodium amaranticolor* Coste et Reyn. as assay host.

Results and Conclusions

The results of test plant experiments (Table 2) showed that the virus disease of *Circaea lutetiana* plants had been caused by the green strain of CMV. It is worth mentioning here that from inoculated *Nicotiana glutinosa* and *N. tabacum* cv. *Bel 61–10*, *N. tabacum* cv. *Samsun*, and *N. tabacum* cv. *Xanthi-nc* plants showing at first systemic symptoms and later recovery the virus could not be passed over to other plants (e.g. *Chenopodium*, *Cucumis*, *Cucurbita* etc.). To maintain

Table 2

Plants susceptible to a virus from *Circaea lutetiana* L.

Hosts	Susceptibility*
Atriplex hortensis L.	L
Atropa bella-donna L.	S
Browallia cordata G. Don.**	L + S
Br. roezli Nichols**	L + S
Br. viscosa H. B. et K.	L + S
Bryonia alba L.	S
Br. dioica Jacq.	S
Chenopodium amaranticolor Coste et Reyn. (Fig. 2B)	L
Ch. quinoa Willd.	L
Cucumis sativus L.	S
Cucurbita pepo L. convar. *patissonina* f. *radiata* Nois. (Fig. 2C)	S
Datura chlorantha Hook.	L + S
D. gigantea hort.	L + S
D. godronii Dan. cv. Minka**	L + S
D. inermis Jacq.	L + S
D. leichardtii F. Muell.	L + S
D. quercifolia H. B. et K.	L + S
D. rosei Safford**	L + S
D. stramonium L. var. *chalybae* Koch **	L + S
D. stramonium L. var. *tatula*	L + S
Gomphrena globosa L. (Fig. 2A)	L + S
Lycium barbarum L.	L
L. carolinianum Walt.**	L
L. chinense Mill.	L
L. flexicaule Pojark.**	L
L. halimifolium Mill.	L
L. horridum Thbg.**	L
Nicotiana chinensis Fisch.***	L + S
N. clevelandii A. Gray	L + S
N. glutinosa L. (Fig. 2D)	L + S
N. knightiana Goodspeed***	L + S
N. megalosiphon Heurck. et Muell.	L + S
N. paniculata L.	L + S
N. sylvestris Speg. et Comes	L + S
N. tabacum L. cv. *Bel 61—10*	L + S
N. tabacum L. cv. *Samsun*	L + S
N. tabacum L. cv. *Xanthi-nc*	L + S
Ocimum basilicum L.	L + S
O. canum Sims	L + S
O. sanctum L.**	L + S
Silene pendula L.	S
Spinacea oleracea L.	S
Tetragonia crystallina L'Hérit.**	L + S
T. echinata Ait.***	L + S
T. tetragonoides (Pall.) O. Ktze	L + S

* Local susceptibility: L, systemic susceptibility: S, local and systemic susceptibility: L + S

** New test plants in plant virology

*** New hosts of cucumber mosaic virus

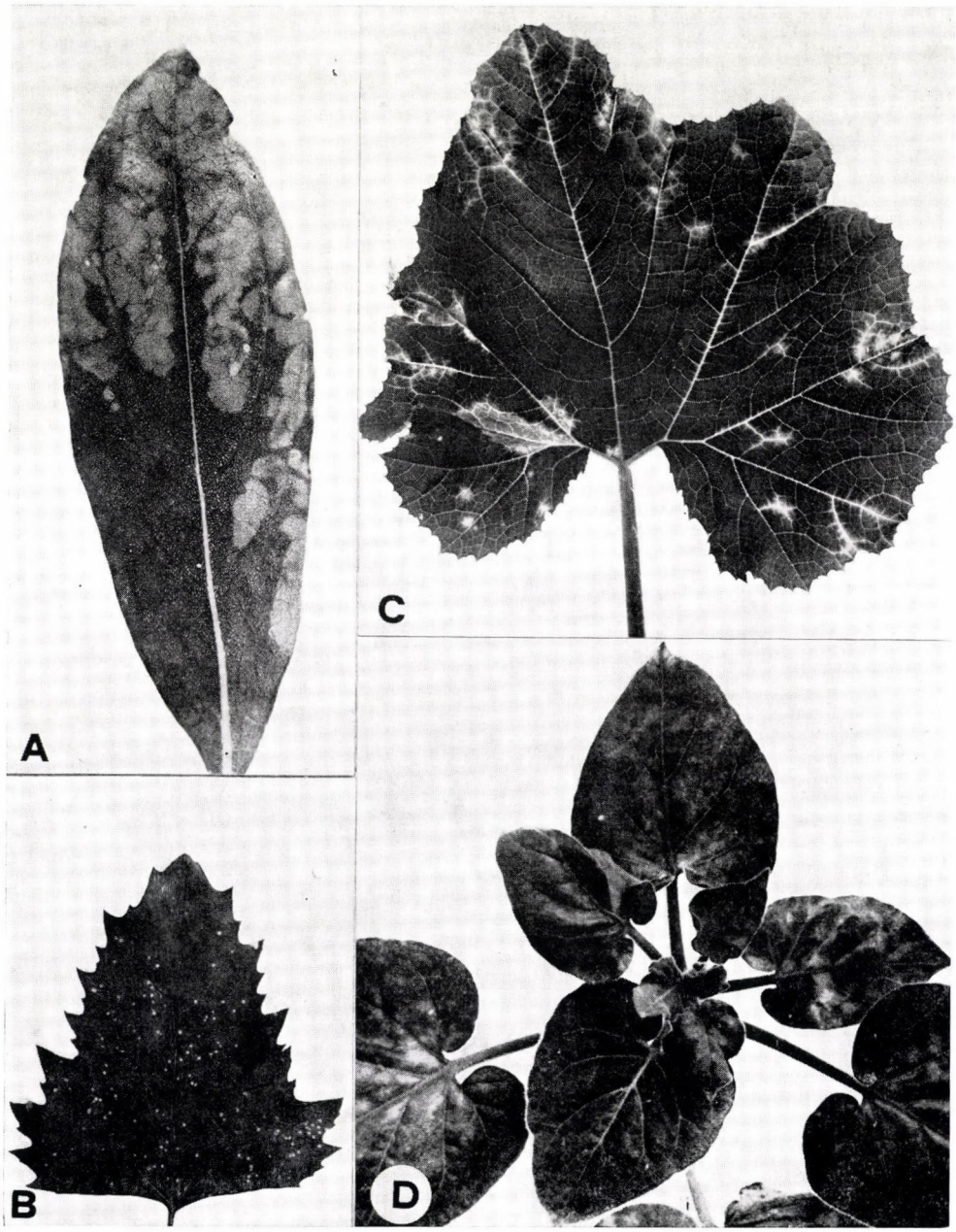

Fig. 2. Symptoms on various test plants inoculated with cucumber mosaic virus from *Circaea lutetiana* L. plants. A: Systemic symptoms on *Gomphrena globosa* L., B: Local lesions on *Chenopodium amaranticolor* Coste et Reyn., C: Systemic symptoms on *Cucurbita pepo* L. convar. *patissonina* Greb. f. *radiata* Nois., D: Systemic symptoms on *Nicotiana glutinosa* L.

and study the Cir-isolate we employed therefore *Cucumis sativus* and *Cucurbita pepo* convar. *patissonina* f. *radiata* plants in the first place.

The Cir-isolate was readily transmitted by *Myzus persicae* in a stylet-borne manner from naturally infected *Circaea* and artificially inoculated *Nicotiana tabacum* cv. *Xanthi-nc* plants. In serological double diffusion tests carried out with sap of diseased *Cucumis sativus* and *Nicotiana glutinosa* leaves, a single precipitation line was observed. The results of the gel-diffusion tests indicated that the Cir-isolate was actually identical with CMV. In the plant protection experiments *Nicotiana tabacum* cv. *Xanthi-nc* plants infected with the Cir-isolate were protected against infection of the subsequently inoculated CMV-W. The results of the plant protection test give evidence of a relationship between the Cir-isolate and CMV-W.

When studying the physical properties of Cir-isolate we found its thermal inactivation point to be at 54—56°C, the dilution end point at 10^{-2}—10^{-3}, and longevity *in vitro* 3 days. These values are lower than those characterizing the physical properties of CMV strains earlier isolated in Hungary (HORVÁTH, 1969; 1973; 1975; HORVÁTH and SZIRMAI, 1973; HORVÁTH *et al.*, 1975a, b).

The demonstration of CMV in *Circaea lutetiana* in Hungary is of special interest. According to our opinion this perennial plant is a highly important virus reservoir, and plays a decisive role in causing the earlier described CMV infections of *Echinocystis lobata* (Michx.) Torr. et Gray. and other, horticultural plants occurring at the same site (cf. HORVÁTH and SZIRMAI, 1973).

The results presented here indicate that *Circaea lutetiana* plants are important natural reservoirs of CMV. This is apparently the first report of a mosaic and leaf deformation disease of *Circaea lutetiana* caused by CMV.

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Influence of Soybean Mosaic Virus Infection on Free Amino Acid Content in Nodules of Soybean (*Glycine max* [L.] Merr.)

By

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The soybean mosaic virus infection influenced the concentration of free amino acid in nodule of soybean plant. The nodules of diseased plant had higher concentration of free amino acid than the nodules of healthy plant. In general, there was an increase in the concentration of different free amino acids in nodules of both healthy and diseased soybeans with the age of the plant throughout the experimental period, but a decrease was observed in the concentration of lysine after 40 days, alanine and serine after 50 days and the glycine after 60 days of virus inoculation.

The synthesis of virus protein by plant tissues almost certainly involves changes in their free amino acid pool, although such changes may be small and transitory (SELMAN *et al.*, 1961). The present investigation has been conducted to see the effect of soybean mosaic virus (SMV) infection on the free amino acid content in nodules of soybean.

Material and Methods

Two lots of 30 plants were taken. One lot of 30 plants was inoculated with SMV at first trifoliate stage and the second lot was kept healthy as control. Nodules from healthy and soybean mosaic virus diseased plants were harvested on 20, 30, 40, 50, 60 and 90 days after inoculation and subjected separately to estimate the free amino acid content by method described by SELMAN *et al.* (1961) with slight modifications.

One gram dried nodule material was crushed in a chilled mortar with 10 ml of 70 per cent alcohol. This was filtered through cheese cloth. This filtrate was centrifuged for ten minutes at 1600 rpm. The supernatant was again filtered. The filtrate was taken and concentrated to about one ml by boiling in water bath. This concentrated solution was used for the estimation of free amino acids using two dimensional paper chromatographic technique (Smith, 1958). Butanol/acetic acid/water (4 : 1 : 5) was used as first solvent and second solvent was phenol/water (4 : 1). Spraying was done with 0.25 per cent ninhydrin solution in acetone. The paper was hanged in chromatographic oven for drying and colour development.

Individual standard amino acids were chromatographed using 0.05 per cent solution of each and their R_f values were calculated. Optical density of the chromatogram was measured by denzitometer. The amount of amino acid was calculated from a standard graph prepared for each amino acid.

Results

The results summarized in Table 1 show that the nodules of infected soybean had higher amount of amino acids than the healthy ones. Seventeen amino acids were detected in nodules of both infected and healthy soybean plants out of twenty one amino acids tested. In general there was an increase in the concentration of different amino acids with the age of the plant throughout the experiment. However, a decrease was observed in the concentration of lysine after 40 days, alanine and serine after 50 days and glycine after 60 days of inoculation in nodules of both healthy and diseased plants.

Table 1

Effect of soybean mosaic virus infection on free amino acid content (mg/g dry wt) in nodules of soybean

Days after inoculation	Treatment	Amino acids						
		Alanine	Aspartic acid	Aspara-gine	Arginine	Citrulline	Cystine	Glutamic acid
20	Healthy	0.20	0.13	0.27	0.11	—	—	0.14
	Diseased	0.29	0.17	0.33	0.18	—	—	0.17
30	Healthy	0.23	0.19	0.34	0.19	—	—	0.18
	Diseased	0.34	0.25	0.37	0.27	—	—	0.24
40	Healthy	0.31	0.23	0.41	0.24	—	—	0.21
	Diseased	0.42	0.27	0.48	0.34	—	—	0.33
50	Healthy	0.34	0.28	0.45	0.31	—	—	0.27
	Diseased	0.46	0.33	0.52	0.46	—	—	0.38
60	Healthy	0.29	0.34	0.50	0.36	—	—	0.32
	Diseased	0.38	0.37	0.55	0.49	—	—	0.44
90	Healthy	0.21	0.39	0.53	0.40	—	—	0.35
	Diseased	0.27	0.42	0.58	0.57	—	—	0.47

Days after inoculation	Treatment	Amino acids						
		Glycine	Histidine	Isoleucine	Leucine	Lysine	Methio-nine	Ornithine
20	Healthy	0.20	0.14	0.12	0.13	0.22	—	0.12
	Diseased	0.27	0.16	0.18	0.22	0.25	—	0.15
30	Healthy	0.24	0.18	0.16	0.17	0.24	—	0.16
	Diseased	0.31	0.21	0.23	0.27	0.29	—	0.19
40	Healthy	0.29	0.23	0.22	0.21	0.30	—	0.20
	Diseased	0.35	0.26	0.27	0.34	0.37	—	0.29
50	Healthy	0.33	0.28	0.26	0.28	0.26	—	0.24
	Diseased	0.42	0.33	0.32	0.38	0.31	—	0.32
60	Healthy	0.36	0.29	0.29	0.32	0.21	—	0.27
	Diseased	0.45	0.35	0.34	0.41	0.25	—	0.35
90	Healthy	0.34	0.31	0.31	0.37	0.19	—	0.29
	Diseased	0.31	0.39	0.37	0.50	0.23	—	0.83

Days after inoculation	Treatment	Amino acids						
		Phenyl-alanine	Proline	Serine	Thre-onine	Trypto-phane	Tyrosine	Valine
20	Healthy	0.13	1.10	1.11	0.59	—	0.12	0.24
	Diseased	0.14	1.24	1.23	0.62	—	0.15	0.33
30	Healthy	0.17	1.14	1.15	0.61	—	0.14	0.27
	Diseased	0.19	1.24	1.28	0.69	—	0.18	0.38
40	Healthy	0.21	1.17	1.18	0.64	—	0.19	0.36
	Diseased	0.24	1.29	1.32	0.74	—	0.22	0.44
50	Healthy	0.26	1.23	1.21	0.70	—	0.23	0.40
	Diseased	0.30	1.34	1.37	0.79	—	0.27	0.49
60	Healthy	0.29	1.27	1.13	0.73	—	0.25	0.42
	Diseased	0.33	1.39	1.30	0.88	—	0.32	0.51
90	Healthy	0.32	1.30	1.02	0.78	—	0.28	0.45
	Diseased	0.36	1.42	1.22	0.92	—	0.37	0.56

Discussion

There are numerous reports on the effect of virus infection on the concentration of free amino acids in leaves of virus infected plants (FIFE and STOKES, 1959; SELMAN *et al.*, 1961; NAMBIAR and RAMAKRISHNAN, 1969 and KAPUR *et al.*, 1974). In the present study also increase in concentrations of free amino acids in nodules of diseased soybean plants have been observed. The accumulation of amino acids in virus affected plants is considered by SELMAN *et al.* (1961) to be indicative of a block in the protein synthesis of plants or of an enhanced amino acid activating system (HAYASHI, 1962). FORD and TU (1969) reported that changes in concentration of free amino acids in virus infected maize were a result of alternation in metabolism resulting from or associated with virus multiplication. The increased concentration of free amino acids in nodules of diseased plants observed in present study can be explained in agreement with this view. An other factor which might have also contributed to increase concentration of free amino acid in nodules of diseased plants is that the utilization of nitrogenous compounds (amino acids etc.) manufactured by *Rhizobium* in nodules may be inefficient because of the added stress on the biological system of supporting SMV-replication, which resulted in an increase in concentration of amino acids and other nitrogenous compounds.

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Investigations on the Antigenic Structure of Fusaria. I. An Electrophoretic Survey of Proteins, Glycoprotein and Lipido-proteino-polysaccharides in the Mycelial Extracts of *Fusarium culmorum* and *Fusarium acuminatum*

By

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A polyacrylamide gel electrophoretic study on the components of potentially antigenic importance present in the crude mycelial extracts of *F. culmorum* and *F. acuminatum* was performed in order to complete the first step of the investigations on the antigenic structure of Fusaria. Most of the fractions separated proved to be glycoproteins, there were some bands of simple proteins also, and a single disc in both extracts was considered to be a lipido-proteino-polysaccharide. Mucopolysaccharides, nucleoproteins and acid mucopolysaccharides were not observed. By comparing the electrophoretic patterns of the two species no major qualitative differences were found. However, the quantitative differences determined in several fraction and the microheterogeneity of the bands as detected by means of a densitometer make it probable, that some fractions will induce specific antibodies.

Since the identification of the members of the genus *Fusarium* may be difficult using traditional mycological techniques some workers attempted the adoption of serological methods. In such a study on *F. oxysporum*, *F. moniliforme* and *F. solani* MADHOSINGH (1964) found, that some degree of serological relationship exists among the three species, and there is a closer relationship between *F. oxysporum* and *F. moniliforme* than between either of these and *F. solani*. Unfortunately, as the author tested the antisera with single isolates of each species his interesting findings do not have an universal validity and it is not clear whether the serological differences determined exist among strains or among species. During the investigations on the serological relationships among eight *Fusarium* species – using again only one isolate of each – SPAAR and VESPER (1970) demonstrated the difficulties of preparing specific antisera. Specific reactions can be obtained in the case only, if disturbing effects of the specific antibodies are minimized. Applying the gel-diffusion method they found, that different reaction times and different dilution grades of antisera and also variegated times in taking blood from the rabbits resulted qualitative differentiations among the fungus species studied. Although their investigations render the use of mycoserological methods more perspective, procedures recommended there, are not easy to reproduce. HORNOK and JAGICZA (1973) prepared a fluorescent antibody reagent for *F. culmorum*. The specificity of the conjugate containing antibodies against the whole-cell antigens proved to be limited: on the basis of the fluorescence intensity *F. culmo-*

rum could be distinguished from several other members of the genus *Fusarium*, however, there were no remarkable differences among strains of *F. culmorum* and *F. graminearum*.

No resounding success in the adoption of serological methods for taxonomical studies was reported either in other fields of agricultural mycology. The main reason of this failure is, that in most cases, when fungi were compared serologically, unexpected cross-reactions without any phylogenetic basis occurred. Presence of these cross-reactions is quite obvious, since the antigenic structure of fungi is a very complicated system and during the immunization procedure or when the serological tests are carried out aspecific members of the above-mentioned complex antigenic mosaic can overtop the specific ones. Consequently, it would be essential to know what components of the fungal antigenic complex induce specific antibodies in the immunized animals. For these investigations the first step seems to be the use of an antigenic material which is more exactly characterized. Therefore, we have made an electrophoretic study of the components of potentially antigenic importance present in the mycelial extracts of *Fusarium culmorum* and *Fusarium acuminatum*. These two species were selected on the account, that the greatest taxonomical problems exist among the members of sections "*Gibbosum*" and "*Discolor*". *F. acuminatum* is a representative of the "*Gibbosum*" and *F. culmorum* seems to be the most characteristic species for the "*Discolor*".

Materials and Methods

Growth of fungi and preparation of mycelial extracts

F. culmorum strain S 120502 and *F. acuminatum* strain L 110401 obtained from the culture collection of this laboratory were investigated. Potato-dextrose solution enriched with 1 g/litre NH_4NO_3 was used for the production of mycelium. The stationary cultures were grown in 500 ml Erlenmeyer flasks containing 60 ml medium, at 25°C, in the dark. Flasks were inoculated with 1 ml spore suspension. According to the different growth intensity of the two species, inoculum concentrations were different: inoculum for *F. culmorum* contained 10^5 conidia/ml, for *F. acuminatum* it contained 2×10^5 conidia/ml. In this manner, cultures of the same physiological condition were obtained after 72 hours' incubation. The mycelium was harvested by filtration, washed three times with distilled water, rolled dry between paper towels and stored at −20°C. The frozen mycelium was grinded mechanically, then was extracted with distilled water for about 3 hours, at 4°C. This suspension was centrifuged at 5000 g for 20 min then at 60 000 g for 60 min in the cold. The protein content of the supernatant was determined by the microbiuret test (BAILEY, 1967) with human serum albumin as the standard. The carbohydrate content was measured by the phenol–sulphuric acid colorimetric method (HODGE and HOFREITER, 1962) using glucose as the reference sugar. The protein concentration in each extracts was adjusted to the same level. The extracts were buffered with Tris-borate buffer before used for further studies.

Polyacrylamide gel electrophoresis and detection of the bands

Gels and buffers were prepared according to MAURER (1971). Separation took place in two layers: the upper of 3.5% acrylamide concentration (5 mm in length) was set between the small-pore gel (7.5% acrylamide) and the spacer layer (a suspension of Sephadex G-200). Separation gels were buffered with Tris-HCl buffer, pH 8.9, the space layer with H_3PO_4-Tris buffer, pH 6.9. The diameter of the gels was 6 mm. Samples containing 400 μg protein in Tris-borate buffer (0.06 M Tris, 0.06 M H_3BO_3, pH 8.6) were subjected to polyacrylamide gel electrophoresis. Electrophoresis was carried out at 4°C using a tank buffer of Tris-glycine, pH 8.3. An electric current of 2 mA/tube was applied, then increased to 4 mA/tube after 30 min and maintained at this intensity until the tracer dye (bromophenol blue) had moved about 60 mm. A run with exchanged electrodes was also performed to determine whether there is any component which migrates toward the cathode.

Proteins were spotted by staining with amido black 10 B (1% in a solution which contained 7.5% acetic acid and 30% ethanol) for 40 min and destaining over several days by rinsing with repeated changes of 7.5% acetic acid. Glycoproteins were stained as described by Caldwell and Pigman (cf. MAURER, 1971) using a modification of the periodic acid – Schiff technique. For lipoproteins method of Ressler et al. (cf. MAURER, 1971) was followed: before electrophoresis one part of saturated sudan black B in ethylene glycol was mixed with two parts of mycelium extract and kept for 36 hours at 4°C. In this case, samples containing 800 μg protein were analysed. Detection of acid mucopolysaccharides was also attempted by staining with 0.1% toluidine blue 0 according to the method outlined by Rennert (cf. MAURER, 1971). Stained gels were photographed on an NC 19 film and scanned by means of a Joyce Chromoscan densitometer.

Results

When mycelial extract prepared from *F. culmorum* was analysed by polyacrylamide gel electrophoresis the following electrophoretic profile was obtained. Remarks concern only the bands detected in the small-pore separation gels after the anodal runs, as neither cathodal fractions, nor bands localized in the large-pore gels were observed. Staining with amido black resulted in 16 disc (in some cases 15) of protein nature. Eight of these (Nos 1, 3, 4, 6, 7, 8, 11 and 12) were major components and constituted almost the total quantity of protein entered the gel. Under our conditions bands 1 and 2, 11 and 10, 12 and 13 migrated close to each other, for this reason fractions 2, 10 and 13 appeared as minor shoulders in the electropherogram (Fig. 1A). Some faint but distinct protein bands were also observed, Nos 5, 9 and 14. They were always present when repeated runs on the same extract were performed but the quantity of these weaker discs as judged

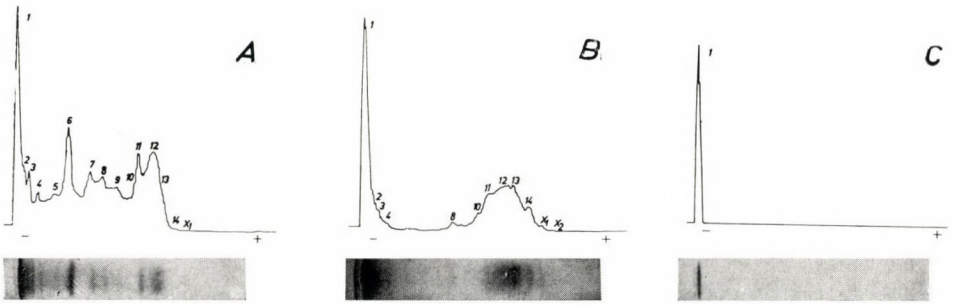

Fig. 1. Disc electrophoretic pattern of mycelial extract of *F. culmorum*: (A) Stained with amido black 10 B; (B) Stained with periodic acid — Schiff reagent; (C) Stained with sudan black B. (Since there were no fractions in the large-pore separation gels these were removed before scanning.)

by the intensity of the staining appeared to vary. The remaining two fractions were not always resolved in our gels. If they were present at all considerable variations in their intensity and in their position were observed with different runs, therefore they are signed in the figures as X_1 and X_2 or X_1 alone. The length of gels differs with staining procedures; staining for carbohydrates resulted in somewhat enlarged gels. There were also small differences in the length of the runs when extracts from the two species were compared. To determine whether two discs are identical or not their position in each gel was calculated as the ratio of the distance moved by the band to the distance moved by the bromophenol blue front and this ratio was designated as the R_f value. Staining for carbohydrates indicated, that six of the eight major components, numbers 1, 3, 4, 8, 11 and 12 were glycoproteins. Bands 6 and 7 should be consider simple proteins, because very faint, almost invisible Schiff positive bands were only observed at their position. Out of the remaining fractions numbers 2, 10, 13 and 14 proved to be glycoproteins, from them Nos 2, 13 and 14 seem to contain high proportion of carbohydrate. Two minor Schiff positive discs (X_1 and X_2) which migrated fast towards the anode were also detected, but their position was not reproducible from run to run. Mucopolysaccharides were not observed (Fig. 1B). In general, the Schiff positive discs were rather lightly stained, due to the low carbohydrate-protein ratio of the extracts. This ratio was 1 : 3 in the case of *F. culmorum*, namely the sample containing 400 μg protein involved only about 130 μg carbohydrate expressed in glucose equivalents. Preliminary experiments showed, that staining with sudan black B resulted in very faint, almost invisible bands, when samples containing 400 μg protein were subjected to gel electrophoresis. This is why the sample volume was doubled for detecting lipoproteins. By the staining method applied a single band was observed in the 7.5% gel at the same position where fraction 1 was found in the case of staining for proteins and for carbohydrates (Fig. 1C). However, it was a question whether patterns obtained by staining after electrophoresis are comparable or not with those obtained by electrophoresis of

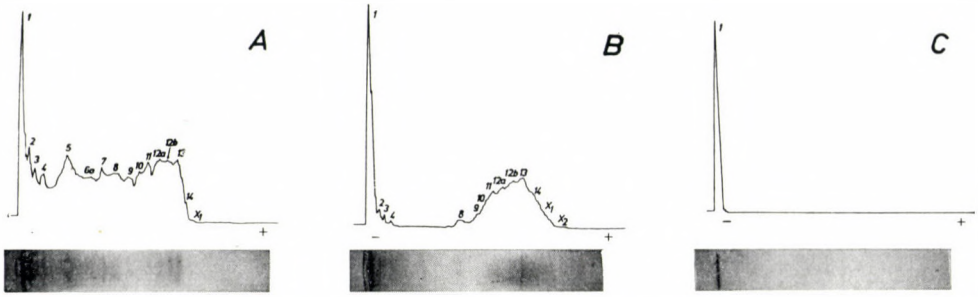

Fig. 2. Disc electrophoretic pattern of mycelial extract of *F. acuminatum*: (A) Stained with amido black 10 B; (B) Stained with periodic acid — Schiff reagent; (C) Stained with sudan black B. (Before scanning the large-pore separation gels were removed.)

a prestained extract. To check this factor of uncertainty some gels used for detecting lipoproteins were counterstained with amido black after electrophoresis. There were no qualitative differences among the protein patterns of these gels and those of presented in Fig. 1A. Therefore it was concluded, that fraction No. 1 is a complex of protein, carbohydrate as well as lipid and should be considered a lipido-proteino-polysaccharide. Acid mucopolysaccharides and nucleoproteins were not present in our gels, since staining with 0.1 % toluidine blue 0 proved to be negative.

In the case of *F. acuminatum* staining with amido black resulted in 16 bands. Nine of these (Nos 1, 2, 3, 5, 8, 11, 12a, 12b, and 13) were major components. One faint disc (X_1) was not characteristic, as great variations in its migration distance and in its intensity were found when duplicate runs were performed (Fig. 2A). After staining for carbohydrates bands 1, 2, 3, 4, 8, 9, 10, 11, 12a, 12b, 13 and 14 were found to be glycoproteins (Fig. 2B). A single fraction No. 1 containing lipid was only observed and this was considered to be a lipido-proteino-polysaccharide (Fig. 2C). Mucopolysaccharides, nucleoproteins and acid mucopolysaccharides were not observed. There were no bands localized in the large-pore gel. Schiff positive discs here were also lightly stained since the carbohydrate-protein ratio was as low as in the case of *F. culmorum*. By comparing the electrophoretic patterns of *F. culmorum* with those of *F. acuminatum* no major qualitative differences were found. Only bands Nos 6 and 6a differed in their electrophoretic mobility. The R_f values were determined as 0.28 and 0.35, respectively. Band 12 with an R_f value of 0.62 present in the electrophoretic profile of *F. culmorum* was resolved into two components, Nos 12a and 12b (R_f values 0.59 and 0.64) when the *F. acuminatum* extract was analyzed. More significant quantitative differences between the two species were observed, especially by comparing the patterns obtained after staining with amido black (Figs. 1A and 2A). Considerable differences were found in the intensity of fractions 5, 10, 11 and 13.

Discussion

According to WEIR (1973) antigens may be protein in nature, or carbohydrate, lipid or nucleic acid they are more frequently complexes such as glycoproteins, lipoproteins, glycolipids and so on. In order to complete the first step of a study on the antigenic structure of Fusaria investigations were carried out on the presence and distribution of these materials in the crude extract of two *Fusarium* species. Most of the fractions separated by means of polyacrylamide gel electrophoresis proved to be glycoproteins, there were some bands of simple proteins also, and a single disc in both extracts was considered to be a lipido-proteino-polysaccharide. Other types of possible antigens were not found in our gels in a detectable amounts. Some of these materials might be involved in the extracts, but they could not migrate in the gels due to their low electrical charge. (Sediment of negligable amount was always found at the edge surface of the spacer layer and large-pore separation gel.)

How many antibodies will develop after applying these extracts for immunization? This number depends on several factors, e.g. on the type of the immunization scheme, on the competition of the antigens, and so on. The most determinative factor however, is the number of the antigens introduced. During our investigations at least 14 (15 in the case of *F. acuminatum*) distinct fractions, potentially antigens according to their chemical nature and molecular weight were found which differ in their electrophoretic mobility. Since the resolution in such studies is limited by the close distribution of the bands over a short length (about 65 mm) this number is probably greater. Scans made by densitometer seem to support this assumption. As can be seen in Figs 1 and 2 considerable microheterogeneity occurred in the fractions.

The proportion of fractions which can induce specific antibodies is open to question also. On the basis of the nearly identical electrophoretic patterns of the two species high proportion can not be expected. However, complete absence of specific antigens is not presumed because of the quantitative differences determined in several fractions as well as the above-mentioned microheterogeneity of the bands. It is also possible, that some fractions proved to be identical by polyacrylamide gel electrophoresis will differ in their antigenic properties. When this article was prepared immunization procedures using extracts of known electrophoretic patterns had already begun. Investigations on the serological activity of fractions separated by this technique will be outlined in the next paper.

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Effect of Temperature on *Fusarium* Root Rot of Pea

By

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Detailed studies have been made on the effect of temperature on *Fusarium* root rot disease of pea. The effect of date of sowing on the disease incidence was observed in two consecutive years. The disease was found to be prevalent in the warmer period of pea growing season; the earlier was the date of sowing the more was the disease incidence. Under controlled conditions soil temperature had significant effect on disease development. The disease-temperature curve was found to follow the pathogen-temperature curve. Both for infection and growth and sporulation of the fungus 30°C was found to be the optimum.

Pea (*Pisum sativum* L.) suffers from a root rot disease caused by *Fusarium solani* f. *pisi* (Jones) Snyder and Hansen. As the pathogen is soil borne, soil temperature plays an important role in disease incidence. Though several investigations on the effect of temperature on disease incidence have been made knowledge is still inadequate. In India pea grows in winter (October to March) when the temperature maximum and minimum is approximately 35 and 2°C respectively. In the fields pea plants were found to get the infection mostly during the warmer periods of the pea growing season. Detailed studies were, therefore, undertaken to see the effect of date of sowing on the disease development as well as the effect of soil temperature on disease intensity under controlled conditions and the effect of temperature on germination of microconidia and growth and sporulation of the pathogen.

Material and Methods

A commonly grown susceptible pea variety Bonneville was used for the experiments. Pure culture of *Fusarium solani* f. *pisi*, isolated from an infected pea plant from I. A. R. I. field was used for all the experiments.

For the experiments on the effect of date of sowing and soil temperature on disease development sand-maize meal medium (180 g dry sand, 20 g maize meal and 60 ml water) was used. Twenty one days' growth at 25°C was used as inoculum. Inoculum was mixed in 10% concentration with field soil, 24 hours prior to sowing and watered lightly so that the pathogen can establish itself in

the soil. The pot experiments were done in 25 cm pots. Ten pots were kept per treatment, with 10 seeds in each pot. Equal number of controls were also kept, where seeds were sown in uninoculated field soil.

As the air temperature of pea growing season in India varies from about 35° to 2°C, an experiment was laid to see if the date of sowing has any effect on the disease incidence. Seeds were sown in inoculated soil at 15 days interval, starting from 1st of October, with the last sowing on the 30th December for two consecutive yeard (1971 and 1972). The number of plants infected up to the pod forming stage was noted. Soil temperature at a depth of 5 cm was recorded daily at fixed hours.

As soil temperature tanks were not available, pots were kept in 20, 25 and 30°C incubator with fluorescent light inside, for studying the effect of soil temperature on disease development. The temperature of the soil was recorded daily. Observations were taken every alternate day from sixth day of sowing up to 32 days. The plants were graded as a) absolutely healthy, b) flaccid and c) completely wilted, according to the degree of infection.

To study the effect of temperature on growth and sporulation, Potato dextrose agar plates, inoculated with 1 mm inoculum bits (24 hrs growth at 30°C) were incubated at 20, 25 and 30°C. The diameter of the circular colonies was measured daily at 24 hour interval. Observations were taken for sporulation separately from the central 1 mm diameters area and the remaining area. As the formation of macroconidia were very poor in all treatments observations were taken only for microconidia.

Microconidial germination was also studied at 20, 25 and 30°C. Spore suspension (90 to 100 conidia per microscopic field) was prepared from 7 days' old PDA cultures and kept in moist chamber for germination at 20, 25 and 30°C. Observations for spore germination was taken after 4, 6, 8, 10, 12, 14, 16 and 20 hours. Number of germinated spores and the length of the germ tubes were recorded.

Results

Date of sowing. Characteristic aerial symptom of *Fusarium* root rot infection is a dark brown lesion at the collar region. The roots also become brown and most of the secondary roots and the primary root get rotted as a result of which the whole plant wilts. For this experiment total wilting of the plants was taken as the disease index. The experiment was repeated for two consecutive years and the data obtained is presented in Table 1.

It is clear from Table 1 that the earlier the date of sowing the more was the disease intensity. Disease percentage was found to increase with increase in air temperature, and consecutively the soil temperature. Maximum infection was observed in both the years when the crop was sown on 1st October. The infection decreased gradually as the sowing was delayed. The plants sown in December were almost free from infection.

Table 1

Effect of date of sowing on root rot incidence by *F. solani* f. *pisi* on pea (var. Bonneville)

Date of sowing	Air temperature in °C (Average of 15 days)				Soil temperature in °C at 5 cm depth (Average of 15 days)				Disease per cent	
	Maximum		Minimum		Morning 7.00 A. M.		Evening 5.00 P. M.			
	'71	'72	'71	'72	'71	'72	'71	'72	'71	'72
1.10	35.0	33.7	18.3	15.3	23.0	21.5	34.0	32.0	80.5	73.3
15.10	30.0	34.7	16.0	17.7	22.0	23.5	28.5	33.5	60.0	64.2
30.10	28.5	30.1	10.5	10.5	16.0	16.5	26.5	28.5	49.5	43.4
15.11	26.7	27.0	9.2	12.0	13.5	17.5	25.5	26.0	23.0	33.2
30.11	25.5	22.5	7.0	8.4	11.0	12.0	22.0	21.0	14.4	12.8
15.12	21.5	22.6	5.4	8.2	9.0	12.0	20.0	21.5	0	5.8
30.12	20.5	19.0	2.4	2.0	7.0	6.0	19.0	18.0	0	0

On analysing the data of the soil temperature it was observed that in the first week of October, when the infection was most severe, the soil temperature maximum and minimum were 34 and 21°C respectively. These temperatures decreased gradually and so also the disease percentage. No infection occurred when the respective figures came down to 21 and 6°C. Maximum temperature remaining the same, when the minimum was 11°C the infection was found to be 12 to 14 per cent.

Soil temperature. The experiment was planned to see if under controlled conditions temperature has any effect on infection and also to find out the optimum temperature for infection. The results are summarized in Table 2.

The data presented in Table 2 show that there is some correlation between temperature and disease development, irrespective of other factors. This pathogen prefers relatively high temperature for infection. Hundred per cent wilting was observed 14 days after inoculation, at 30°C while the respective figure was less than 10 per cent at 20°C.

It was also found that at higher temperature the state of final symptom, that is, wilting was accomplished more rapidly than that at lower temperature. Most of the plants at 25 and 30°C wilted within 2 days, whereas at 20°C the plants took mostly 4 days or sometimes even more for complete disease development.

Growth and sporulation of the pathogen. The growth and sporulation of the pathogen in PDA at different temperatures are presented in Table 3. The data presented in Table 3 show that both mycelial growth and sporulation has some correlation with the temperature. The fungus preferred the higher temperature (30°C) for both growth and sporulation. The growth rate at 25°C was found to be little less in the initial stage as compared to 30°C. But after 5 days the rate

Table 2

Effect of soil temperature on the infection of pea (var. Bonneville) plants by *F. solani* f. *pisi* as indicated by number of plants at different stages of infection

Age of plants in days	Soil temperature in °C								
	20 Plant condition			25 Plant condition			30 Plant condition		
	Healthy	Flaccid	Wilted	Healthy	Flaccid	Willted	Healthy	Flaccid	Wilted
6	100	0	0	100	0	0	51	42	7
8	100	0	0	64	36	0	34	17	49
10	91	9	0	40	24	36	16	19	65
12	80	20	0	20	22	58	0	14	86
14	69	22	9	17	4	79	0	0	100
16	69	11	20	0	17	83	0	0	100
18	58	22	20	0	0	100	0	0	100
20	50	8	42	0	0	100	0	0	100
22	47	11	42	0	0	100	0	0	100
24	0	58	42	0	0	100	0	0	100
26	0	51	49	0	0	100	0	0	100
28	0	47	53	0	0	100	0	0	100
30	0	33	67	0	0	100	0	0	100
32	0	0	100	0	0	100	0	0	100

increased and became equal to that at 30°C. At 20°C the fungus grew very slowly in the initial stage after which even though its rate increased, the growth was much lesser in comparison to that at higher temperatures.

Sporulation was much more influenced by the higher temperature (30°C) than the mycelial growth. At that temperature the sporulation started much earlier and at a very high rate. Sporulation at 30°C was about four times as that at 25°C, whereas only slight change was observed in the rate of growth which was also nullified later. In all cases, the formation of microconidia was found to be affected by temperature. Macroconidia production which was significantly less always was not affected by any temperature. Central portion (1 mm diameter area), which consisted of the inoculum bit was observed to cut much more number of spores at all temperatures.

Germination of microconidia. Data on germination of the microconidia and growth of the germ tubes at different temperatures are presented in Table 4. From the results in Table 4 it is evident that the germination of the spores is influenced by temperature. The spores at 25 and 30°C started germinating after 4 hours incubation, while those at 20°C started only after 10 hours. Again, the pattern of rate of germination was found to be more or less similar at all the temperatures; the rate increased for sometime, attended the maximum value and then decreased again. Though initially the germination started late at 20°C, the rate of germination was observed to be higher at that temperature in comparison to

Table 3

Effect of temperature on radial growth and sporulation of *F. solani* f. *pisi*

Period of growth in days	Incubation temperature in °C	Mycelial growth		Sporulation	
		Diameter of colony in (mm)	Area of colony in sq. mm	No. of microconidia in central 1 mm diameter area	No. of micro-conidia/sq. mm of the remaining area
1	20	5	19.63	44	0
	25	7	38.5	44	0
	30	12	142.4	460	0
2	20	12	142.4	440	0
	25	25	491.0	450	1
	30	30	706.9	1120	7
3	20	24	531.0	2600	1
	25	40	1257.0	4300	10
	30	44	1452.0	9000	34
4	20	36	1018.0	2700	2
	25	56	2464.0	4300	16
	30	60	2828.0	10000	60
5	20	46	1689.0	2700	9
	25	66	3422.0	4400	17
	30	70	3850.0	10000	60
6	20	60	2828.0	2700	10
	25	86	5813.0	4400	17
	30	86	5813.0	10000	60

that at higher temperatures. Among the three temperature treatments, there was not much difference in the total number of spores germinated ultimately after 20 hours, and though the initial starting time was much different the trend through the later period of the three was almost the same.

Discussion

Among several environmental factors, soil temperature is the one which has significant effect on disease development as regards soil borne pathogens. Until and unless the temperature is favourable most of the pathogens can not infect the hosts, even if other factors are congenial. Under controlled conditions the soil temperature studies revealed that both for total infection and early appearance of the disease 30 °C was the optimum, followed by 25 °C. At 20 °C the disease appeared late and more time was required to complete the infection.

The effect of soil temperature on the root rot infection may be due to its direct effect on growth and sporulation of the pathogen or its effect on one or more steps in the series of complex physiological and biochemical processes in

Table 4

Effect of temperature on the germination of microconidia of *F. solani* f. *pisi*

Incubation period (hours)	Incubation temperature in °C	Spore germination %age	Percentage of spore having germ tube length of				
			1—16 μ	17—48 μ	49—80 μ	81—112 μ	More than 112 μ
4	20	0	0	0	0	0	0
	25	3	3	0	0	0	0
	30	8	8	0	0	0	0
6	20	0	0	0	0	0	0
	25	10	9	1	0	0	0
	30	31	21	10	0	0	0
8	20	0	0	0	0	0	0
	25	35	26	9	0	0	0
	30	55	29	19	7	0	0
10	20	12	12	0	0	0	0
	25	57	14	33	10	0	0
	30	69	20	28	15	6	0
12	20	52	44	8	0	0	0
	25	66	13	30	13	10	0
	30	75	18	25	12	18	2
14	20	73	35	29	9	0	0
	25	75	25	20	13	14	3
	30	81	26	20	11	11	12
16	20	75	20	24	14	3	0
	25	76	13	26	12	15	10
	30	85	14	27	20	9	15
20	20	80	10	35	20	15	0
	25	81	7	30	23	10	11
	30	84	9	22	29	12	12

the host or pathogen or in both of them due to their interaction during disease development. In general, along with other microorganisms the fungi also require specific temperatures for growth and sporulation, which were also in decreasing order from 30 to 20 °C in this pathogen. This confirms the earlier works (MOORE, 1923; MANNS and ADAMS, 1924; DAVIES, 1944; LOCKWOOD, 1962) where the optimum temperature for the growth of this fungus was observed to lie between 25 and 28 °C. Sporulation was significantly more in the central 1 mm portion than in the remaining area, and was not much affected by temperature. This consisted of the inoculum bit which was a day older than the rest and was initially at 30 °C. The chain of metabolic reactions responsible for spore production can be assumed to be much ahead in this case due to the initial higher temperature.

The disease–temperature curve often follows wery closely the pathogen–temperature curve (WALKER, 1969). For this isolate also the temperature favourable for infection and that for growth and sporulation of the pathogen was found to

coincide. At favourable temperature the pathogen grows very rapidly in the soil thus increasing the inoculum potential, as well as dissemination of the disease from place to place thus increasing the disease incidence.

The percentage of spore germination was not affected very much by temperature. Though the lower temperature initiated germination late, ultimately no difference was observed in the percentage of spore germination. It may be suggested that the metabolic processes controlling germination of spore are slowed down by low temperature but are not inhibited.

Date of sowing was observed to affect the disease incidence; the earlier was the sowing, the higher was the disease incidence. The air temperature during the pea growing season in India varies approximately between 35 and 2 °C. On analysing the soil temperature and infection percentage during a particular period, a definite correlation was observed to exist between these two. Relatively warmer air and soil temperature of October favoured the disease development. In the U. S. A. also the disease was found to be more prevalent in warmer periods of May and June (STONE, 1923; MANNS and ADAMS, 1925; DRECHSLER, 1925; SCHREUDER, 1951). During the present studies it was observed that when the maximum temperature remained constant at 21°C, variation in minimum temperature caused difference in disease intensity. When the minimum temperature was 12 °C, the diseases percentage was 5.8 and it was nil when the minimum temperature was less than 9 °C; while 100 per cent infection occurred at a constant temperature of 20 °C, though the process of infection was much slower in comparison to that at the higher temperatures.

Based on the observations made by these experiments it may be suggested that avoidance of sowing of this crop in relatively warmer period during October will help in reducing the disease incidence. The late sown varieties may be thus more suitable as they will escape the disease.

Acknowledgement

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New Records of Powdery Mildews on Certain Ornamental Plants

By

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The paper describes new powdery mildews on *Muehlenbeckia complexa* and on *Solanum pseudo-capsicum* which have not been recorded before in the available literature. Two other powdery mildews on Stock and Poplar are new to Egypt. The *Oidiopsis* state of China Aster powdery mildew was observed for the first time.

Powdery mildews of wire-plant (*Meuhlenbeckia complexa* Meissn.) and Jerusalem cherry (*Solanum pseudo-capsicum* L.) have not been recorded before in Egypt or elsewhere as far as the available literature is concerned.

Powdery mildew of stock (*Matthiola incana* R. B.) and poplar (*Populus* sp.) are not recorded in Egypt (ASSAWAH, 1969; EL-HELALY *et al.*, 1966 and YUSEF, 1964). Powdery mildew of stock incited by *Oidium matthiolae* had been recorded from Palestine (RAYSS, 1940), and that of Poplar incited by *Uncinula salicis* was recorded from the U. S. A. (REESE, 1939), from Canada (BRODIE, 1945), from Japan (ITÓ, 1959) and from Spain (DIAZ, 1963).

Powdery mildew of China Aster (*Callistephus chinensis* Nees) incited by *Oidium* state was recorded from Egypt (ELAROSI and ASSAWAH, 1959) from U. S. A. (CHILDS, 1940), from Greece (EMMANOUIL, 1970), from Palestine (RAYSS, 1940), but the occurrence of the *Oidiopsis* state on China Aster plants seems to be the first observation in Egypt or elsewhere in the available literature.

Powdery mildews of wire-plants, poplar, China Aster were observed during the Spring of 1974, and those of Jerusalem cherry and stock during the summer of the same year.

Powdery mildew of *Muehlenbeckia* appeared on both sides of the affected leaf-like stems, as white powdery patches which sometimes coalesced occupying an appreciable area (Fig. 1A). Conidiophores are erect, septate, $5-7\ \mu$ in diameter and up to $140\ \mu$ long. Conidia are borne singly, hyaline, dimorphous, cylindrical and obclavate, the former being predominant; $35-53\times14-21\ \mu$ (av. $44.2\times16.5\ \mu$) the latter $46-53\times14\ \mu$ (av. $50.2-14.0\ \mu$) (Fig. 2,1). These characteristics agree fairly well with those reported by BROOKS, 1953, and MUKERJI, 1968, for *Oidiopsis taurica* (Lév.) Salm. The authors are inclined to refer the *Muehlenbeckia* powdery mildew fungus to the conidial state of *Leveillula tauric* (Lév.) Arn.

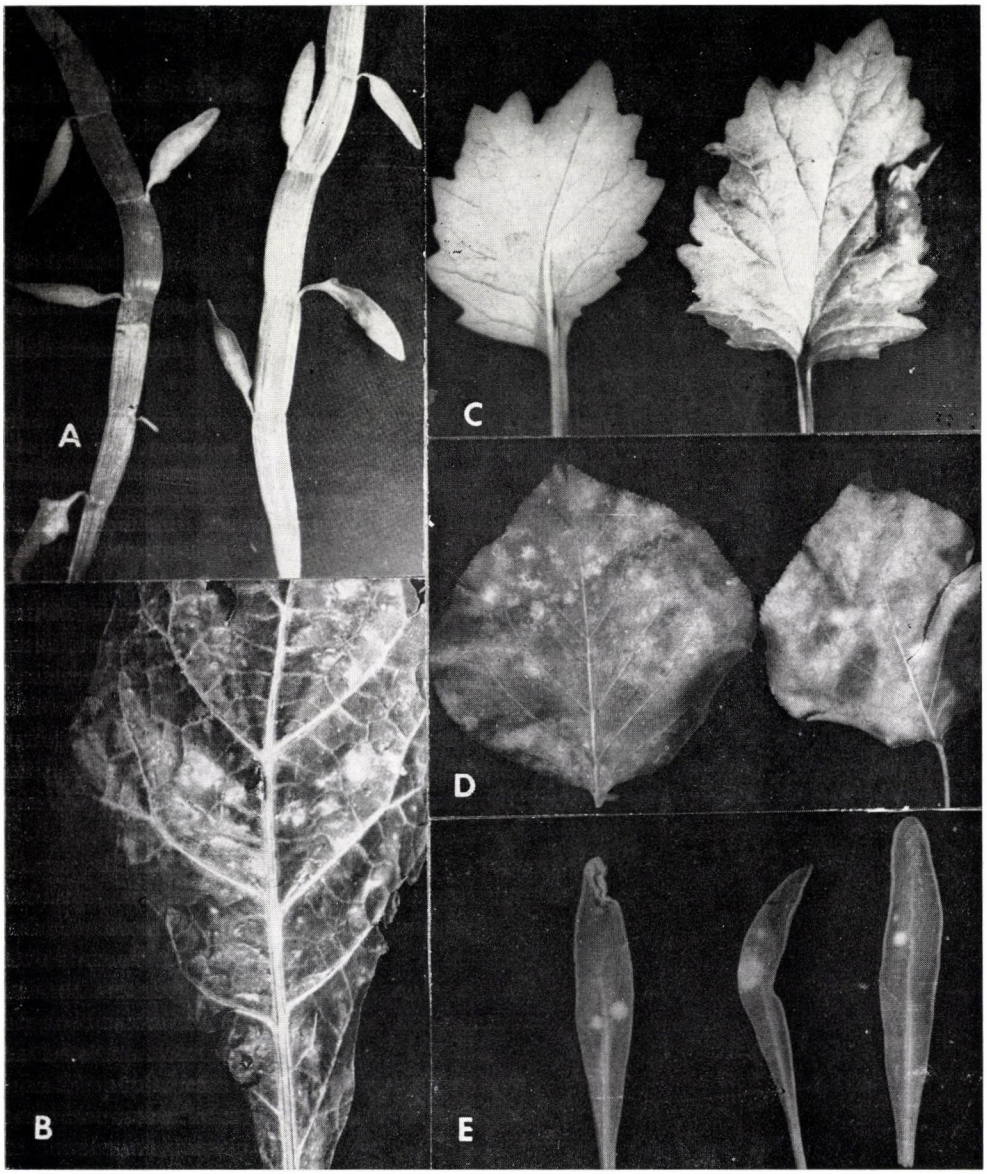

Fig. 1. Symptoms of powdery mildew of: (A) Wire-plant, (B) Jerusalem Cherry, (C) China Aster, (D) Poplar, (E) Stock

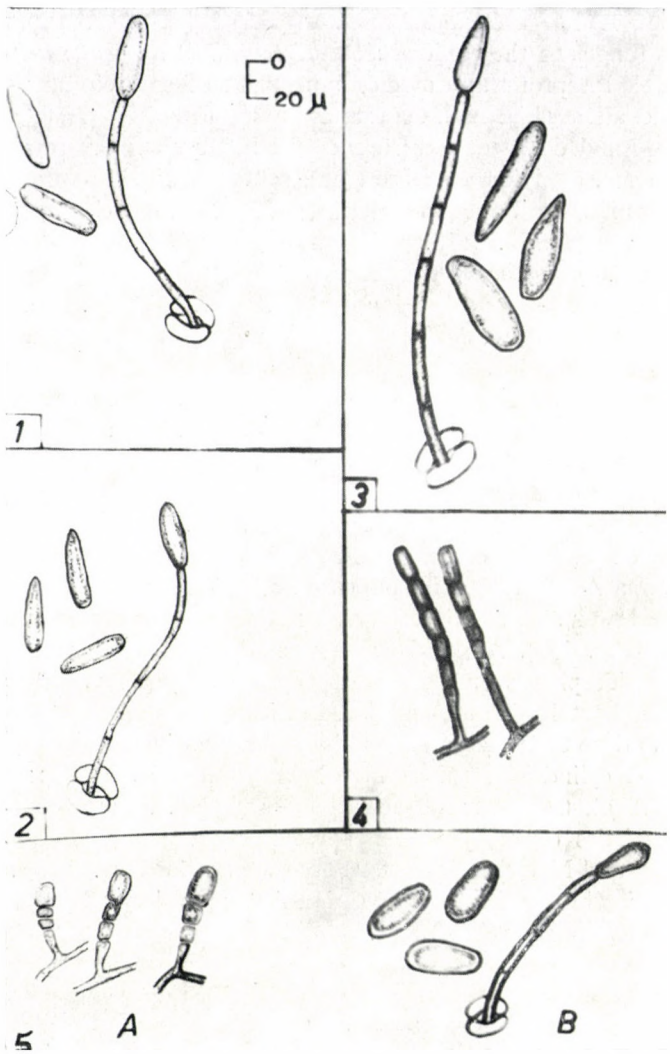

Fig. 2. Conidiophores and conidia of powdery mildew of: (1) Wire-plant, (2) Jerusalem Cherry, (3) China Aster, (4) Poplar, (5) Stock

On Jerusalem cherry, symptoms appeared first as yellow spots on the upper leaf surface with corresponding white sparse growth on the lower side (Fig. 1,B). The affected leaves became curled, brown and eventually fall down. Conidiophores emerged from the endophytic mycelium through the stomata of the leaf. Conidiophores are erect, septate 3.5 μ in diameter and up to 140 μ long. Conidia are borne singly, hyaline, obclavate, $49-74\times14-25$ μ (av. 60×19 μ) (Fig. 2,2). These characteristics are in agreement with those reported by BROOKS, 1953,

and MUKERJI, 1968, for *Oidiopsis taurica* (Lév.) Salm. The authors are inclined to refer this fungus to the conidial state of *Leveillula taurica* (Lév.) Salm.

On stock, symptoms appeared on both leaf surface as circular, white patches which often coalesce (Fig. 1,E). On examining the causal organism, two imperfect states, *Oidium* and *Oidiopsis* were found, the latter was more predominant. For the *Oidium* state, conidiophores are erect, septate, 3.5 μ in diameter and up to 35 μ long. Conidia are borne in long chains, hyaline, barrel-shaped to ellipsoid $18-28\times9-14$ μ (av. 20.7×10.4 μ). For the *Oidiopsis* state, conidiophores are erect, septate, 2.6 μ in diameter and up to 123 μ long. Conidia are borne singly, hyaline, obclavate $35-46\times14-21$ μ (av. 38.6×17.2 μ) (Fig. 2,5). The presence of the two imperfect states, *Oidium* and *Oidiopsis* on this host plant has not been observed before and no other examples in the literature have been encountered.

On poplar, symptoms appeared on both leaf surfaces as white, circular or irregular patches which became confluent (Fig. 1,D). The affected leaves turned yellow and became dry. Conidiophores are erect, septate $5-6$ μ in diameter and up to 33 μ long. Conidia are borne in long chain, hyaline, barrel-shaped to ellipsoid, $21-32\times11-14$ μ (av. 26×13.8 μ) (Fig. 2,4). These characteristics agree well with the *Oidium* state of the powdery mildew fungi (YARWOOD, 1957).

On China Aster, symptoms appeared on both surfaces of the leaves especially on the lower side as white patches which often coalesced covering considerable area (Fig. 1,C). Affected leaves turned brown and eventually fall prematurely. Conidiophores emerged from the endophytic mycelium through the stomata of the host leaf to the exterior. Conidiophores are erect, septate, $4-7$ μ in diameter and up to 193 μ long. Conidia are borne singly, hyaline, dimorphous, obclavate and cylindrical, the former being predominant, $32\times68\times14-18$ μ av. 61.4×16.5 μ), the latter $49-63\times14-18$ μ (av. 58.3×16.8 μ) (Fig. 2,3). The characteristics of the pathogen agree fairly well with those reported by BROOKS, 1953, and MUKERJI, 1968, for *Oidiopsis taurica* (Lév.) Salm. The authors are therefore inclined to identify the China Aster powdery mildew to the conidia state of *Leveillula taurica* (Lév.) Arn.

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Fungitoxicity of Phytoalexins Derived from Potato Against Mycelial Growth of *Phytophthora infestans*

By

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Rishitin, phytuberin, lubimin, solavetivone and solavetivenone, phytoalexins from potato tuber were tested against mycelial growth of the causative microorganism of the late blight disease. All the phytoalexins but lubimin significantly inhibited the mycelial growth of *Phytophthora infestans*. Although these compounds are not primary determinants of plant disease resistance, still they could be useful as chemicals in plant disease control, according to their antifungal activity.

Of the phytoalexins isolated so far from potato tubers infected with *Phytophthora infestans* and other fungi or bacteria, significant accumulations of rishitin, lubimin (lyubimin) and phytuberin were observed in the resistant host–parasite interaction (TOMIYAMA *et al.*, 1968a; VARNS *et al.*, 1971; METLITSKII *et al.*, 1971). These terpenoid compounds distinctly inhibit the germ tube formation of zoospores of *P. infestans* and, as the quantity required for inhibiting the fungal development accumulates within a relatively short time, they are assumed to be primary factors in disease resistance.

Two new sesquiterpenes have recently been isolated from potato tubers infected by *P. infestans*, these materials accumulated not only in the incompatible but also in the compatible interaction (COXON *et al.*, 1974). The accumulation of these compounds *i.e.* of solavetivone [(spirovetiva-1(10), 11-dien-2-one); possibly the same as katahdinone (ZACHARIUS *et al.*, 1974)] and solavetivenone (spirovetiva-1(10),3,11-trien-2-one) is higher in the compatible than in the incompatible host–parasite combinations (ZACHARIUS *et al.*, 1974; BECZNER *et al.*, 1975).

Data on the fungitoxic effect of the phytoalexins above, referring only to the zoospore germination or germ tube elongation of *P. infestans*, cannot give a satisfying explanation of the role of phytoalexins in either spontaneous or acquired resistance. In addition, the sensitivity of *P. infestans* to chemicals *e.g.* antibiotics varies according to the different stages within its life cycle (ÉRSEK, 1975). The much debated role of phytoalexins in disease resistance, and the peculiar sensitivity of *P. infestans* to some chemical agents suggests the need for additional data on the fungitoxic effect of these postinfectional compounds.

Materials and Methods

Investigations on the inhibition of mycelial growth of *P. infestans* by phytoalexins were performed with race 1.2.3.4. The fungus inoculum was cultured on a modified Henniger solid medium at 19 °C for seven days.

The phytoalexins rishitin, phytuberin, lubimin, solavetivone and solavetivenone, metabolic products of infected potato tubers, were used in the experiments. Phytoalexins were dissolved in 96% ethanol to get a concentration of 100 μg/ml in Henniger-medium. Levels of 50 μg/ml and 25 μg/ml were obtained by further dilutions with the medium. Each dilution was adjusted with ethanol to a final alcohol concentration of 1.90%. The control medium contained the solvent only, in appropriate concentration. The purified phytoalexins used were kindly supplied by Dr. D. T. COXON (Food Research Institute, Norwich, UK).

For each phytoalexin a 5-mm-diameter plug, cut from the perifery of a 7-day-old culture of *P. infestans*, was placed in the centre of a 6.2-cm-diameter Petri-dish containing 2.5 ml Henniger medium with the given concentration of phytoalexin in it. The cultures were incubated at 19 °C until growth of control plates had reached almost to the edge. Measurements were then made of the radius of the colony less that of inoculum plug, and the mean effective dose value (ED_{50}) for each tested compound was calculated.

Results and Discussion

The fungitoxicity of five phytoalexins, naturally occurring in infected potato tubers, was observed, as regards the mycelial growth of *P. infestans*. Figures 1 and 2 show that all the phytoalexins investigated inhibited the fungal growth. In Figure 3 the inhibition of mycelial growth is demonstrated as a function of phytoalexin concentration. Correlation between the concentration of a given phytoalexin and the inhibition of hyphal growth was more or less linear, the inhibitory effect, however, varied greatly according to the phytoalexin.

Rishitin, solavetivone, and solavetivenone had significant inhibitory effects on fungal growth even in the low concentration of 25 μg/ml; phytuberin was less active, and the lubimin showed fairly low antifungal activity even at a concentration of 100 μg/ml.

The extent of mycelial growth was measured at each phytoalexin concentration, and the ED_{50} values were calculated (Table 1). It seems that the mycelial growth is more sensitive to rishitin than is the zoospore germination. The ED_{50} value for rishitin using zoospore germination test, is 2.4×10^{-4} *M* (TOMIYAMA *et al.*, 1968b), and in the case of mycelial growth as presented here is 1.5×10^{-4} *M*. An even lower ED_{50} value (4×10^{-5} *M*) for mycelial growth was reported by WARD *et al.* (1974). Solavetivone and solavetivenone have almost the same effect as rishitin on mycelial growth but no data are available concerning their effect on zoospore germination. Mycelial growth exhibited similar sensitivity to phytu-

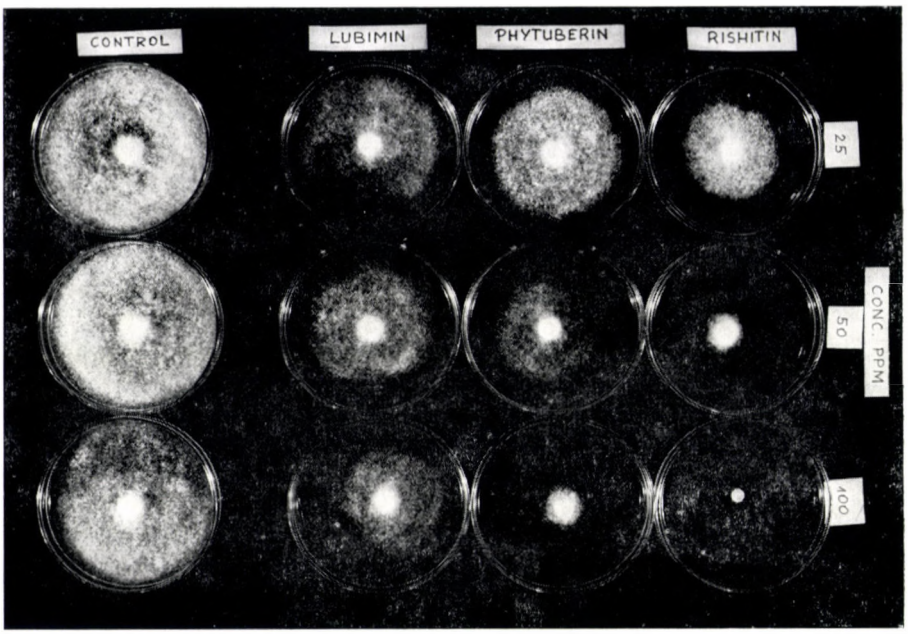

Fig. 1. Growth of *Phytophthora infestans* race 1.2.3.4 on culture medium containing rishitin phytuberin or lubimin of different concentrations

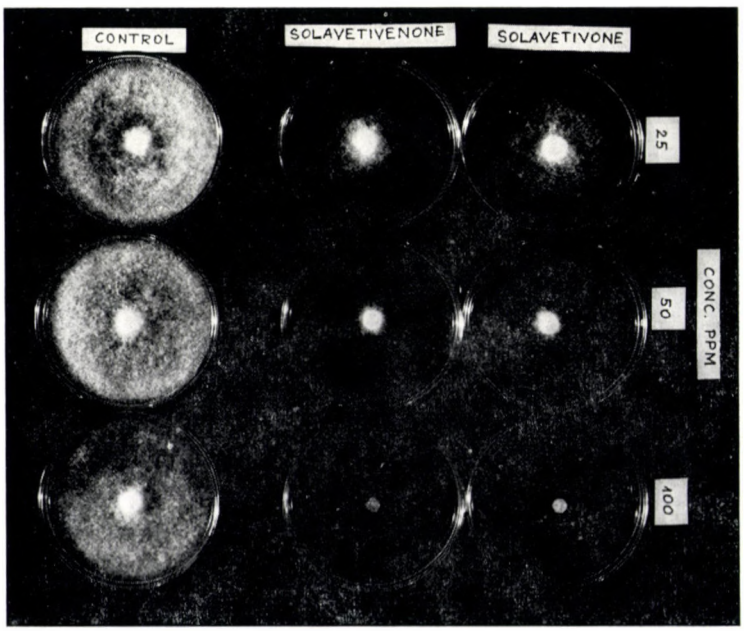

Fig. 2. Growth of *Phytophthora infestans* race 1.2.3.4 on culture medium containing solavetivone or solavetivenone of different concentrations

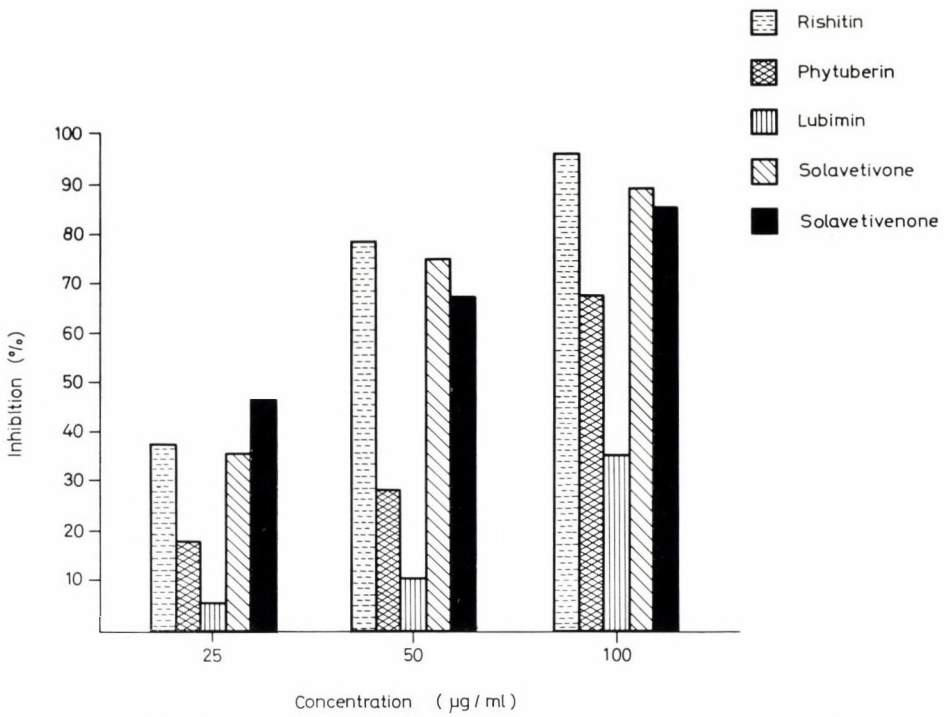

Fig. 3. Inhibition of mycelial growth of *Phytophthora infestans* race 1.2.3.4 by phytoalexins obtained from potato tuber

berin (ED_{50}; $2.7 \times 10^{-4} M$) as did zoospore germination [ED_{50}; approx. $2.4 \times 10^{-4} M$, calculated from VARNS' (1970) results]. Lubimin exhibited strikingly low effect on mycelial growth (ED_{50}; $6.8 \times 10^{-4} M$) compared to its activity against zoospore germination, where the ED_{50} was $1.9 \times 10^{-4} M$ (METLITSKII and OZERETSKOVSKAYA, 1973).

Of the results obtained the most surprising was the high activity of solavetivone against the mycelial growth of *P. infestans*. This compound was detected in very high amounts in the compatible interaction and, at the same time, the mycelium spread over in tubers (18 days after inoculation, using a whole tuber inoculation method). Additionally, other phytoalexins were not detected or were present only in low concentrations (BECZNER *et al.*, 1975). It is difficult to explain how the mycelium can grow over the tubers in the presence of such an active compound.

The data presented here indicate that of the phytoalexins tested, rishitin, phytuberin, solavetivone and solavetivenone can be considered as effective fungicides against *P. infestans*. There are suggestions to use this type compound in plant disease control (WARD *et al.*, 1975). Hence, the activity of the phytoalexins against *P. infestans* may be of great importance due to possible inhibition of

Table 1

Effect of phytoalexins on the mycelial growth of *Phytophthora infestans* race 1.2.3.4

Phytoalexin	Concentration (μg/ml)	Colony growth[1,2] (mm)	ED_{50} ($M \times 10^4$)
Rishitin	25	18	1.5
	50	6	
	100	1	
Phytuberin	25	23	2.7
	50	20	
	100	9	
Lubimin	25	27	6.8
	50	25	
	100	18	
Solavetivone	25	18	1.4
	50	7	
	100	3	
Solavetivenone	25	15	1.4
	50	9	
	100	4	
Control		28	

[1] Radius of colony less the radius of inoculum plug
[2] Mean of two experiments with two replicates each

mycelial growth within plant tissues, as well as the formerly demonstrated inhibition of zoospore germination. For it is generally not known at what stage of the infection the chemicals are applied to the field.

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Increased Production of Some Amino Acids — A Possible Mechanism for Mercury and Captan Tolerance by Fungicide-adapted Isolates of *Macrophomina phaseoli*

By

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Mycelial mats of an isolate of *Macrophomina phaseoli* and mercury and captan tolerant isolates of *M. phaseoli* were analysed paper chromatographically and thirteen amino acids, namely DL-aspartic acid, L-glutamic acid, DL-serine, glycine, L-tyrosine, L-hydroxyproline, DL-alanine, L-histidine, L-arginine, DL-valine, DL-B. phenylalanine, leucine and iso-leucine were detected from all the three isolates. Of these histidine and glutamic acid were present in much greater quantities in the mycelia of mercury tolerant and captan tolerant isolates of *M. phaseoli* respectively. When histidine was added in medium supplemented with mercury the growth of *M. phaseoli* was greatly enhanced, indicating probably detoxification of the fungicide by the amino acid. Similarly, addition of glutamic acid and histidine in captan supplemented medium enhanced the growth of *M. phaseoli*.

Inactivation of toxicants by fungal metabolites have been reported by several workers. A review of the effect of metabolites on the antimicrobial agents has been made by GOTTLIEB (1957) who reported that the metabolites that reverse the toxicity of antimicrobial substances include amino acids, purines, phospholipids, growth promoters, organic acids, nucleic acids, enzymes, fatty acids, alkaloids and sugar derivatives. The reversal of the activity of a large number of fungicides and antibiotics by amino acids have been reported by a number of workers (BAILEY and CAVALLITO, 1948; ZENTMYER and RICH, 1956; ZENTMYER *et al.*, 1960; ASHWORTH and AMIN, 1964). A mercury and a captan tolerant isolate of *Macrophomina phaseoli* were developed in this laboratory by repeated transfers of the fungal isolates on progressively increasing concentrations of the respective fungicides. Studies were undertaken on the chromatographic analysis of the amino acids of the mycelial mats of the fungicide adapted as well as the parental isolates of *M. phaseoli*, as well the effect of some amino acids on the growth of *M. phaseoli* in fungicide supplemented media.

Materials and Methods

The fungicides used were Ceresan Wet (2.5% mercury as methoxymethy mercury chloride) and Captaf (83% N-trichloromethylthio-4-cyclohexene, 2-dicarboximide-Captan).

A culture of *Macrophomina phaseoli* (Mauble.) Ashby was isolated from stem rot infected jute plant (*Corchorus capsularis* L.). From this original culture a mercury tolerant isolate and a captan tolerant isolate were obtained by repeated transfers of the isolates on progressively higher concentrations of Ceresan Wet and Captaf respectively. These three isolates of *M. phaseoli* were used in the present studies.

For amino acid analysis the mycelial extracts of *M. phaseoli* were prepared as described by SINGH (1972) with some modifications. The isolates were grown in Richards medium (liquid) in 250 ml Erlenmeyer flasks for 10 days and the mycelia were extracted by filtering through filter papers. The mycelia were then hydrolysed with 6 N HCl by autoclaving at 15 psi for 30 minutes. A little of $SNCl_2$ was added to each material to avoid the humin formation. The hydrolysed materials were filtered through filter papers and 1 ml of the filtrates were centrifuged at 2000 rpm for 30 minutes. The clear solutions thus obtained were used for analysis of amino acids.

Paper chromatographic analysis of the mycelial extracts for amino acids were made according to the method described by BLOCK *et al.* (1958) using Whatman No. 1 papers. The solvents used were phenol-ammonia-water (80 : 3 : 20 v/v) and *n*-butanolacetic acid-water (4 : 1 : 5 v/v) for the two directions. The chromatograms were sprayed with 0.25% ninhydrine in *n*-butanol. The spots of the amino acids were developed in an incubator at 70°C. The identity of the compounds was compared with standard compounds run on another chromatogram simultaneously.

For studying the effect of histidine and glutamic acid on the growth of *M. phaseoli* in fungicide amended media, the amino acids were added to Richards agar in Erlenmeyer flasks (melted and held at 40°C) supplemented respectively with Ceresan Wet (50 ppm) and Captaf (100 ppm). 20 ml of the media were poured in each of the 10 cm diameter petri plates and allowed to solidify. The centre of each plate was inoculated with a 6 mm disc of *M. phaseoli* (original culture). Three replications were kept for each treatment. The inoculated petri plates were kept at 27°C and records on growth were taken from 3rd day onwards upto 7 days and the linear growth of the isolates per day in each treatment was calculated.

Results

Amino acid compositions of the mycelia of original and fungicide tolerant isolate s of M. phaseoli

Paper chromatographic analysis of the mycelia of the original as well as mercury and captan adapted isolates of *M. phaseoli* demonstrated the presence of thirteen amino acids, viz., DL-aspartic acid, L-glutamic acid, DL-serine, Glycine, L-tyrosine, L-hydroxyproline, DL-alanine, L-histidine, L-arginine, DL-valine, DL-phenylalanine, leucine and iso-leucine in all the three isolates (Table 1).

Table 1

Amino acids in the mycelia of original and mercury and captan tolerant isolates of *Macrophomina phaseoli*[1]

Amino acids	Original isolate	Mercury tolerant isolate	Captan tolerant isolate
DL-Aspartic acid	+	+	+
L-Glutamic acid	trace	++	++++
DL-serine	+	+	+
Glycine	+	+	+
L-Tyrosine	+	+	+
L-Hydroxyproline	+	+	+
DL-Alanine	+	+	+
L-Histidine	trace	++++	++
L-Arginine	+	+	+
DL-Valine	+	+	+
DL-P. phenylalanine	+	+	+
Leucine	+	+	+
Iso-Leucine	+	+	+

Trace +, ++, ++++ represent comparative amounts.

[1] Relative amounts of amino acids are recorded on the basis of spot density on the chromatogram.

Of these L-glutamic acid and L-histidine was produced only in traces by the original isolate, but much increased production of these two amino acids were recorded in the mycelia of mercury and captan tolerant isolates of *M. phaseoli*. Among the fungicide adopted isolates, of L-histidine production was more in mercury adapted isolate, while L-glutamic acid production was more in captan adapted isolate of *M. phaseoli*.

Effect of L-histidine and glutamic acid on the growth of Macrophomina phaseoli in media containing mercury (Ceresan Wet) and captan

The media were amended respectively with 50 ppm Ceresan Wet and 100 ppm captaf which were sublethal to *M. phaseoli*. All the fungal isolates were strongly inhibited by 200 ppm of both histidine and glutamic acid, as such 150 ppm of the compounds were added to the fungicide amended media. As will be seen from Table 2, much increase in the growth of *M. phaseoli* in Ceresan Wet amended medium was recorded when histidine was added to the medium. Addition of glutamic acid did not have any appreciable effect. An increase in growth was also noted when both histidine (75 ppm) and glutamic acid (75 ppm) were added, but the effect was a little lesser as compared to addition of histidine (150 ppm) alone.

In captan supplemented medium on the other hand, the growth of *M. phaseoli* was increased with the addition of either histidine (150 ppm) or glutamic

Table 2

Effect of L-histidine and glutamic acid on linear growth of *Macrophomina phaseoli* in media containing Wet Ceresan (50 ppm) and Captaf (100 ppm)[1]

Treatment	Per day linear growth (in mm)
M. phaseoli + Ceresan Wet (control)	3.54
M. phaseoli + Ceresan Wet + L-histidine (150 ppm)	17.25
M. phaseoli + Ceresan Wet + glutamic acid (150 ppm)	3.57
M. phaseoli + Ceresan Wet + L-histidine (75 ppm) + glutamic acid (75 ppm)	16.50
M. phaseoli + Captaf (control)	5.31
M. phaseoli + Captaf + L-histidine (150 ppm)	13.75
M. phaseoli + Captaf + glutamic acid (150 ppm)	23.75
M. phaseoli + Captaf + L-histidine (75 ppm) + glutamic acid (75 ppm)	23.75

[1] Per day linear growth is the mean of three replications over the growth for 7 days.

acid (150 ppm) but the effect of glutamic acid was much more pronounced. Growth was also increased when histidine (75 ppm) and glutamic acid (75 ppm) were added in combination.

Discussion

The present studies indicated some differences in the intracellular amino acid pools in the mycelial mats of the original isolate of *Macrophomina phaseoli* and the mercury (methoxymethyl mercury chloride) and captan adapted isolates. Thirteen amino acids could be detected in all the isolates, but their amounts varied. Histidine was produced in much greater amount in mercury tolerant isolate while the amount of glutamic acid was much greater in captan tolerant isolate. Following a survey of the literature on the effects of a wide variety of toxicants on various bacteria and fungi, Gottlieb was able to list some modes of detoxification of antimicrobial compounds by the metabolic products of the microorganisms, amino acids being one of the important metabolic products. According to ASHWORTH and AMIN (1964) cystein and glutathione, the amino acids having free SH groups, protected *Aspergillus niger* against mercury poisoning, the mechanism of tolerance being explained as due to a pool of intracellular SH that is free of proteins and that protects enzyme systems by forming complexes with Hg as it is taken up by the thallus. The mechanism, however, may not be similar for all the fungi. Thus, KIESSLING (1961) reported that *Chaetomium globosum* and several other fungi were not protected against mercury by SH. In the present study also cystein or glutathione were not detected in the mycelial mats of

M. phaseoli, but an excess production of histidine over the original isolate was recorded in the adapted isolate. In the present case, therefore, the ability of the mercury adapted isolate of *M. phaseoli* to grow in higher doses of mercury appears to be due to detoxification of Hg by binding of the metal with histidine. ALBERT (1952) found that histidine was one of the strongest chelators of amino acids he tested for their ability to bind several metals. An external application of histidine in mercury supplemented medium also greatly enhanced the growth of original isolate (non adapted) of *M. phaseoli*. The reversal of copper-8-quinolinolate toxicity to *Aspergillus niger* and *Botryosphaeria ribis* by histidine have also been reported by ZENTMYER, RICH and HORSFALL (1956).

Lukens and Sisler (1958) in their studies on the fungitoxicity of captan to *Saccharomyces pastorianus* observed that the sulfhydryl compounds cysteine, homocysteine, glutathione, coenzyme A and thioglycerol showed appreciable antagonistic action to captan if added before or simultaneously with the fungicide. According to them cystein reacts with captan *in vitro*, the product of the reaction being cystine, tetrahydrophthalimide, H_2S, CS_2, 2-thiazolidinethione-4-carboxylic acid and HCl. They also observed that although captan is not capable of reacting with other than sulfhydryl groups, trichloromethylthio group or thiophosgene released from captan by sulfhydryl groups was apparently capable of reacting with amino hyroxyl, sulfhydryl and possibly other groups and histidine and serine reacted with thiophosgene to form ultraviolet absorbing compounds.

The present studies indicated a greater production of histidine and glutamic acid in captan adapted isolate of *M. phaseoli* and antagonistic effects of both the amino acids to captan when applied simultaneously in culture medium. But the exact mechanism involved in the detoxification process is, however, not very clear. Further studies are in progress to find out the possible mechanisms involved.

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Apoplexy of Apricots

V. Pathological and histological investigations of the apoplexy of apricots

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The authors carried out histological investigations with apricot grafts which showed signs of apoplexy. They used apricot grafts which were infected by *Pseudomonas syringae* and *Cytospora cincta*.

Infection caused by bacteria produced significant quantities of resin both in the xylem, phloem and bark as well as in the cells (tracheae and fibre cells are excluded). Resinlike substance was formed in shapes of droplets. As a result of the infection the cell walls grew in thickness and almost became double that of the healthy cells.

Gummosis is more intensive as a result of the infection caused by bacteria. The appearance of the gum was not in the form of droplets or shapes of a sphere, but in mass in the phloem. Blocks of gum were formed in the xylem, tracheae and fibres. *Cytospora*-infection did not produce growth in the cell wall.

These results proved that with the help of histological investigations, there is a chance to differentiate between apoplexy and dying off caused by bacteria and *Cytospora*.

Investigations which have been carried out since 1967, have proved that the apoplexy of apricots in Hungary is caused by two pathogens, namely *Cytospora cincta* Sacc. and *Pseudomonas syringae* Van Hall. Apoplexy of apricots is developed by the separate or joint infection of these two pathogens (KOVÁCS, 1970; KLEMENT *et al.*, 1972; ROZSNYAY and KLEMENT, 1973).

Disease which developed from infection of these two pathogens under laboratory conditions showed similar symptoms on the observed apricot samples. Many other scientists have been carrying out experiments concerning the pathological histology in the field for etiological investigation of the apoplexy of apricots (BEREND, 1959; BABOS, 1970; STIEBER and BABOS, 1967). In these experiments they used samples obtained from trees which had died off long ago. They had no idea about the pathogen which made these trees die. The objective of the study was to carry out histological investigations for comparing results, in which the two pathogens causing the diseases were well known. For this reason twigs infected by *P. syringae* and *C. cincta* were examined.

Methods

For histological investigations two and three years old apricot grafts were used. The samples were inoculated under laboratory conditions by *C. cincta* and *P. syringae*, at predetermined intervals. Samples were inoculated using methods that were previously described (KLEMENT *et al.* 1973; ROZSNYAY and KLEMENT, 1973). The samples used for histological investigations were obtained from tissues infected under laboratory conditions.

Samples PF_1, PF_2 and PF_3 came from trees that were inoculated by *P. syringae* in November 1973. These samples were examined and evaluated in January 1974, soon after symptoms appeared. Sample CF_1 was inoculated in November 1972, samples CF_2 and CF_3 came from wood infected by *C. cincta* in November 1973. All of them were examined in April 1974.

We took extra samples for control experiments. These samples, namely K_1, K_2, K_3 were selected in accordance with the age and physiological state of the original sample.

The histological investigation of the bark and wood of untreated and infected apricot grafts were carried out in the usual way (SÁRKÁNY and SZALAI, 1964). The samples were boiled in an autoclave under pressures of 4–5 atmospheres and in a mixture of water and glycerol with a ratio of 1 : 1. Later, they were desiccated as they passed through a series of alcoholic solutions. The different stages of investigations consisted of: encasing in paraffin, section cutting of a microtome, preparation of samples and finally the microscopic examination, measuring and taking of photos of sections.

We used toluidin-blue and methylene-blue (coloring agents) with 3% of concentration, diluted in alcohol with 50% concentration.

Results

Examination of apricot grafts infected by P. syringae

The objective of examination of grafts infected by bacteria was to reveal some of the changes in the tissue of epidermis (bark + phloem) and of the xylem and to make comparisons with samples uninfected. The cells of phloem and bark tissues contain gum-resin droplets. The cell walls grow in thickness and become almost double of the healthy tissues. The epidermis and the layer that comes next is loose and shows tendency to break up (Figs 1 and 2).

The microscopic photos of Figs 3 and 4 demonstrate well the difference between the thickness of walls of sieve tubes and parenchyma cells (which make up the phloem) and the resin droplets accumulating along the cell walls.

As a result of infection caused by bacteria, resin droplets appear in the tissues which make up the xylem. Resin droplets were observed only in the cells of

the ray, longitudinal parenchyma in the xylem, which are responsible for the transportation of nutrients and storing. The cell walls that make up the above mentioned tissues were significantly greater in thickness than the walls of untreated samples. However, tracheae and fibres, which make up the xylem did not show any sign of change (Figs 5 and 6).

As a result of comparative investigations, the conclusion was that infection caused by bacteria within the xylem as well as in the phloem and bark brought about intensive resin formation. We have to point out that resin formation is in the form of droplets and thus is different from resin formation observed in case of *Cytospora* infections as we will have a chance to observe it.

The picture of ray-parenchyma shows the shapes of resin droplets, as a result of the infection (Figs 7, 8, 9 and 10). We were unable to detect any signs of bacteria present using microscopic examinations since most probably they broke up during the process of boiling of the samples in an autoclave.

Examination of apricot grafts infected by C. cincta

As the infection of fungus in the phloem tissues develops to an advanced stage, the cells get completely necrotized and saturated with resinlike substance. Mycelium could not be detected, but its presence is most obvious, since the reproductive structures of the fungus i.e. pycnidiums containing conidia developed on the outer part of the necrotized bark. Histological investigation of pycnidium formed in the layer near the surface of the bark proved that their structure is made up of grains (Fig. 11). The chamber-like structures of pycnidia of the samples taken from the cross-section are well seen (Fig. 12).

As a result of infection caused by *Cytospora*, resin accumulates. Resin formation to such an extent in the case of infection caused by bacteria did not occur. Therefore, due to the infection, the resin formation and the resin quantity in the cells varied from the quantities observed in the cells of samples infected by bacteria (Figs 13, 14 and 15).

The resinlike material was not only present in the ray, longitudinal parenchyma but also in the tracheae and in the grains (Fig. 14). BANKO and HELTON got similar results in 1974. They carried out histological experiments in which they used one year old seedlings of peach infected by *C. cincta.*

Resin formation in this case was not in the form of droplets or did not have shapes of a sphere but occurred in mass in the cells from which the above-mentioned tissue-like materials are built up (Figs 13, 14 and 15). The mycelium of the fungus can be well seen in the tracheae of the xylem.

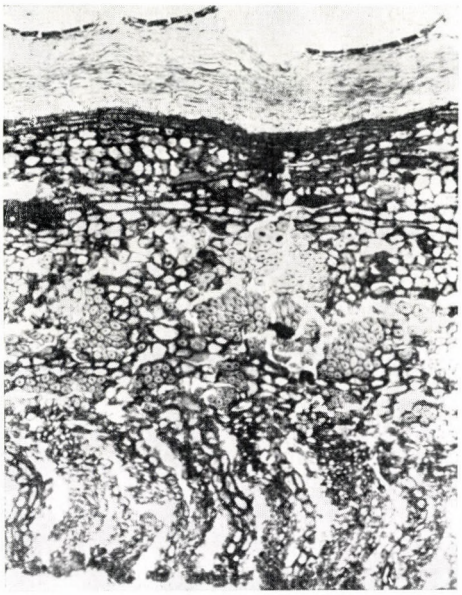
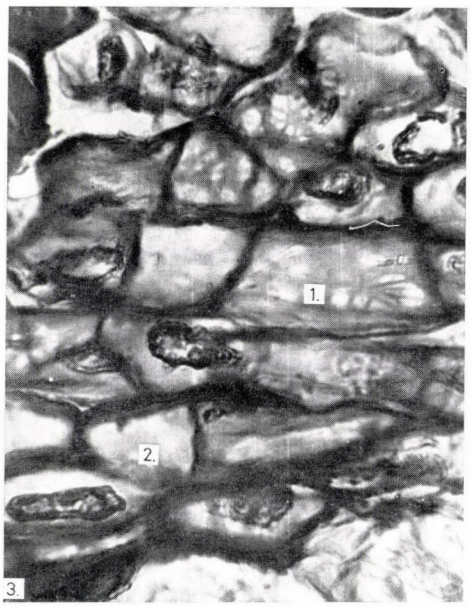


Fig. 1. K_1 is the cross-section of lignified apricot bark, phloem and xylem, general view. Collected: 8. January 1974. Magnification: ×90

Fig. 2. PF_3 is the cross-section of lignified apricot bark, phloem and xylem, general view. Collected: 8. January 1974. Magnification: ×90



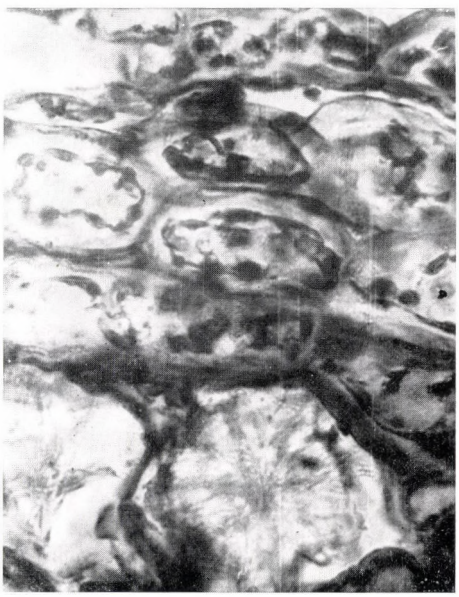
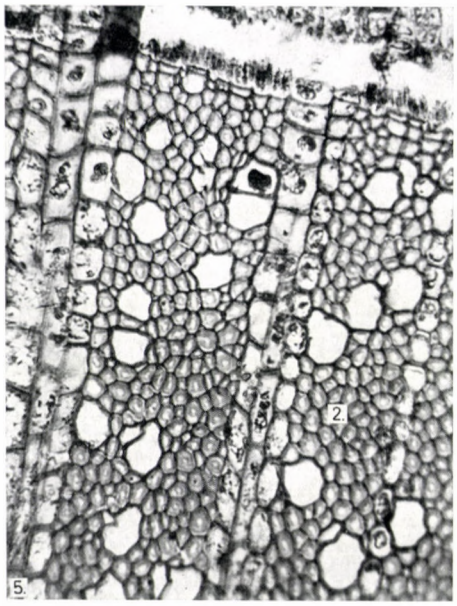


Fig. 3. K_3 is the cross-section of lignified apricot barkphloem. The thin walls of sieve tubes (1) and the longitudinal parenchyma (2) cells can be well recognized. Collected: 8. January 1974. Magnification: ×600

Fig. 4. PF_1 is the cross-section of lignified apricot phloem. The tick walls of longitudinal parenchyma cells and the droplets of gum within the cells can be well recognized. Collected: 8. January 1974. Magnification: ×600



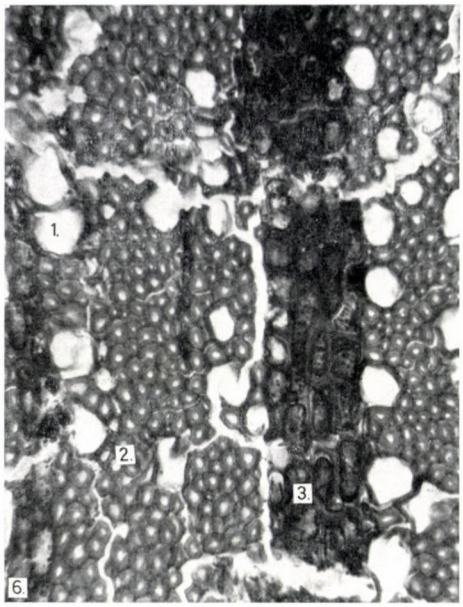


Fig. 5. The cross-section of lignified apricot xylem is marked as K_1. There are no droplets present in the cells of the rays (3). (1) Tracheae (2) fibres. Collected: 8. January 1974. Magnification: × 300

Fig. 6. The cross-section of lignified apricot xylem is marked as PF_2. The walls of the ray cells are thick (3). Droplets of gum can be seen in the cells. Collected: 8. January 1974. Magnification: × 300

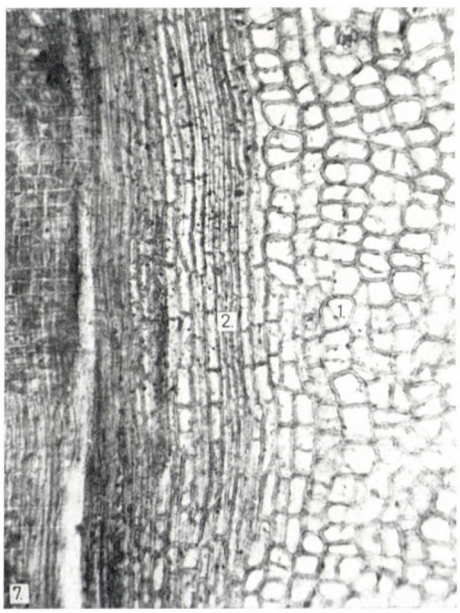


Fig. 7. K_2 is the lignified ray-parenchyma of apricot (1) and the primary cells (2) vertical section. Collected: 8. January 1974. Magnification: × 120

Fig. 8. PF_2 is the vertical section of lignified apricot ray parenchyma and primary wood cells with droplets of gum. Collected: 8. January 1974. Magnification: × 120

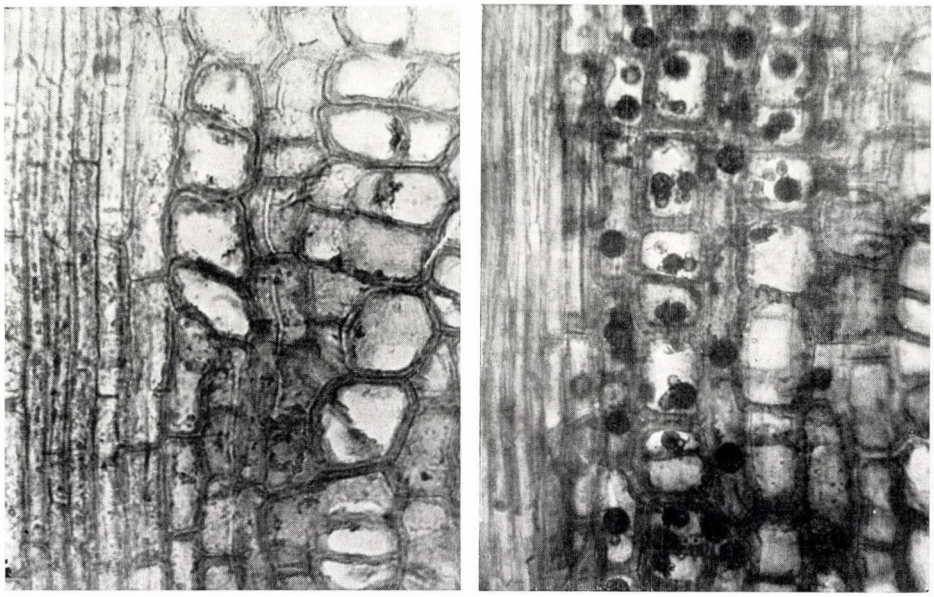


Fig. 9. K_2 is the lignified ray parenchyma and the primary cells which contain the gum. Vertical section. Collected: 8. January 1974. Magnification: ×300

Fig. 10. The lignified ray parenchyma and the primary cells containing the gum are marked as PF_2. Vertical section. The walls of ray-parenchyma cells, which grew in thickness can be recognized, fairly well. Collected: 8. January 1974. Magnification: ×300

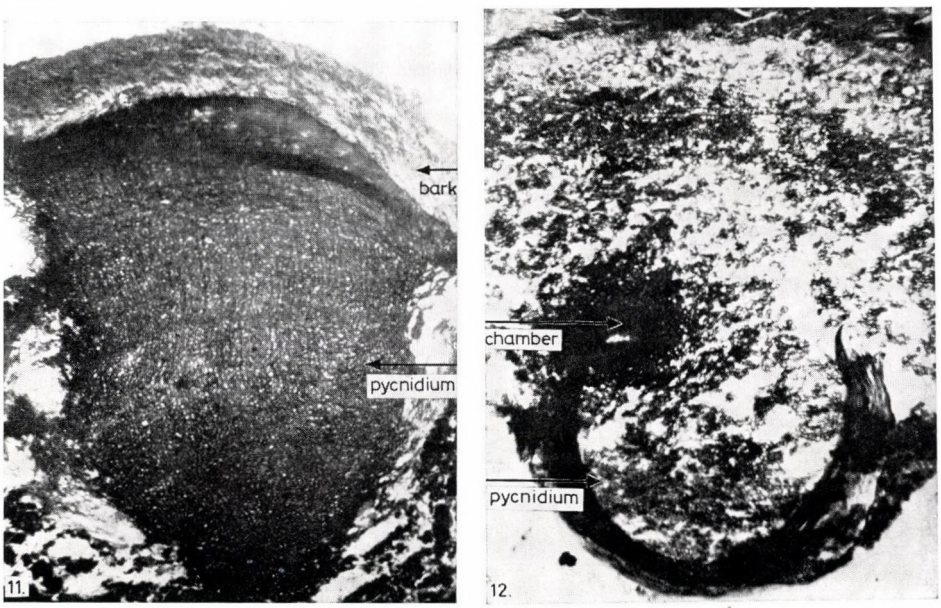


Fig. 11. CF_1 is the vertical section of pycnidia of *Cytospora cincta*. The fibres, which make up the structure of the pycnidia are well seen. Collected: 12. September 1973. Magnification: ×90

Fig. 12. The cross-section of the pycnidium of *Cytospora cincta* with chamberlike structure. Collected: 12. September 1973. Magnification: ×90

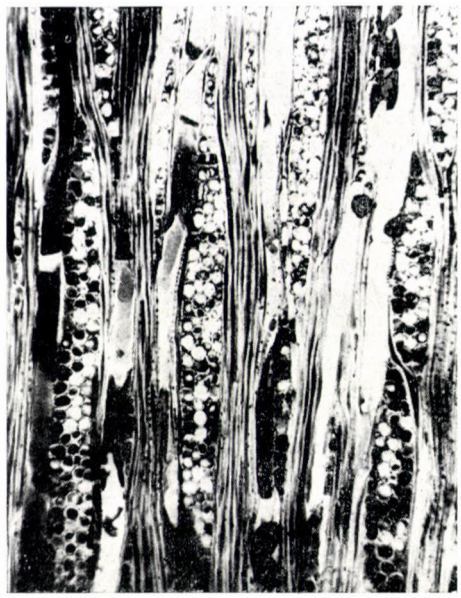


Fig. 13. CF_2 is marked as the vertical section of lignified apricot grafts infected by *Cytospora cincta*. The gum-material can be seen in the ray cells and tracheae. Collected: 30. April 1974. Magnification: × 120

Fig. 14. The vertical section of apricot grafts infected by *Cytospora cincta* is marked as CF_3. The gum can be traced along the ray-cells and tracheae. Collected: 30. April 1974

Fig. 15. The vertical section of apricot grafts (lignified) infected by *Cytospora cincta* is marked as CF_2. Gum in the ray parenchyma and primary cells is well detected. Collected: 30. April 1974. Magnification: × 120

Fig. 16. CF_2 is the basic hympha of *Cytospora cincta*. Vertical section. Gum-material is present in the fibres along the tracheae and in the longitudinal parenchyma cells. Collected: 30. April 1974. Magnification: × 600

Discussion

When our objective was to study the spread of bacteria in the grafts of apricot, we found that bacteria in the xylem spread fast in the longitudinal direction, while this longitudinal progress cannot be observed in the tissues of parenchyma or if there is, it takes place very slowly (ROZSNYAY and KLEMENT, unpublished results). Histological investigations give satisfactory explanation for the understanding of previous observations and of the development of the disease. According to these experiments we are allowed to make the conclusion that bacteria get into the wood up to the xylem through injuries (on the surface of wood) where they are able to get to the medulla-parenchyma, through the passages of ray parenchyma.

The spread of bacteria in the intercellulars of the medulla-parenchyma and the primary wood components in longitudinal direction is certain. Transversial spread is also possible through the ray parenchyma, starting out from a point which is located further on. Approximately 20—25 cm far from the center of infection the cambium gets infected through this tissue and the infection may get into the phloem. The spread of bacteria in the longitudinal direction takes place in the phloem tissue through the xylem. Histological investigations proved that so many bacteria do not enter into the tracheae. This is the explanation for the observation in which bacteria did not bring about periodical infections however, the spread of the infection is influenced by the exterior environment and physiological conditions and thus occurs locally. Progress of the spread of bacteria in the intercellular passages of the ray-parenchyma which surround the tracheae is also possible, but we have no proof for this.

It is very difficult to determine precisely which pathogen brought about the die back. However, with the help of histological investigations we are able to make difference between the die back caused by bacteria or *Cytospora.* In case of infection caused by bacteria the cell walls of the sieve tubes of phloem, the cell walls of ray-parenchyma-xylem, furthermore the cell walls of ray-parenchyma grow in thickness, while these are not true in case of infection caused by bacteria. The other characteristic of infection caused by bacteria is the droplet shape of resinlike material, found in cells. In the case of infection caused by the fungus, cells are full of resinlike substance. Similar difference appeared as a result of the tracheae, which did not become saturated with resin. However, at the same time, frequent resin blocks were formed in the tracheae due to injuries caused by the fungus.

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Role of Phenolics in Bacterial Blight Resistance in Cotton

(Short Communication)

By

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Bacterial blight of cotton caused by *Xanthomonas malvacearum* (Smith) Dowson is one of the most potential diseases in many cotton-growing countries of the world. The biochemical changes induced in nature in resistant and susceptible strains of cotton have not been critically studied, although some phenolics in their oxidised forms have been implicated in resistance mechanisms in number of other crops (COLE and WOOD, 1961; FARKAS and KIRÁLY, 1962). Direct inhibition of pectolytic enzyme activity by oxidized catechins, leucoanthocyanins and oxidized phenols have been suggested as possible mechanisms conferring resistance (COLE and WOOD, 1961). In order to elucidate the biochemical attributes of resistance or susceptibility, shifts brought about in the total phenolics and leucoanthocyanin content of resistant and susceptible varieties of cotton "in health" and under the stress of bacterial infection were studied in the present investigations.

Two varieties of cotton known for their resistance and susceptibility traits, viz. G-27 (*Gossypium arboreum*) and H-14 (*Gossypium hirsutum*) respectively (JALALI and SINGH, 1971) were selected for these studies. Four samples of fresh leaves of same physiological age from each lot were crushed, extracted with ethanol 3–4 times and filtered. The supernatant was refluxed in ethanol at 80°C for ten minutes and filtered. The pooled filtrates of each sample were passed through a column of activated charcoal to remove chlorophyll, then filtered and volume made to 50 ml. The resultant extract was stored in a cool chamber till further analysis. For total phenolic estimation an aliquot of 0.1 ml of ethanolic leaf extract was diluted to 7 ml and 0.1 ml Folin-Ciocalteau reagent then added and thoroughly shaken for about five minutes. 1 ml of saturated sodium carbonate was added after shaking and volume made to 10 ml by thorough mixing. The absorbance of the blue-coloured complex was measured spectrophotometrically at 725 mμ. Total phenols were quantified on the basis of a standard curve prepared by using quericitrin as a standard.

For leucoanthocyanin analysis, an aliquot of 0.1 ml ethanolic extract under test was mixed with 5 ml of the "reagent A" (77 mg $FeSO_4.7H_2O$ in 100 ml *n*-buta-

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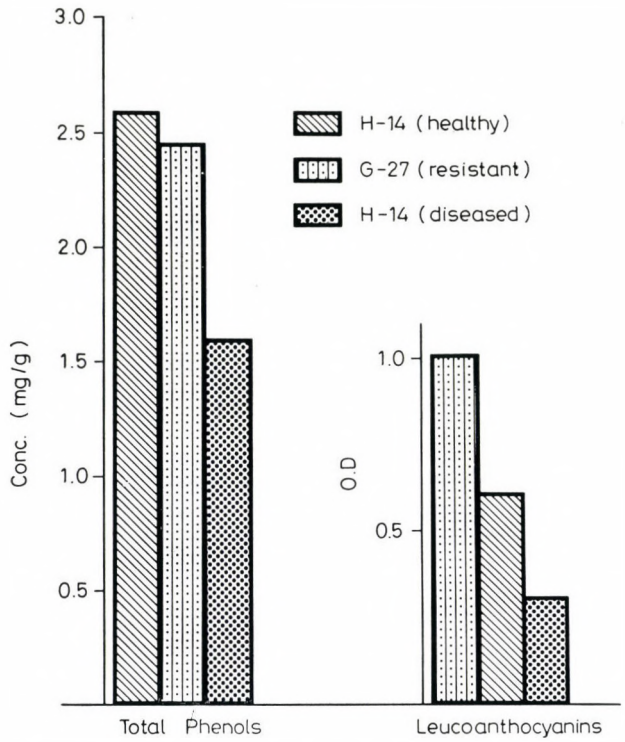

Fig. 1. Total phenols and leucoanthocyanin shifts in resistant and susceptible varieties of cotton to *Xanthomonas malvacearum* infection

nol mixed with 40% HCl in 3 : 2 ratio) in large tube and maintained at 35 °C on water bath for 15 minutes; the tube was loosely fitted by a glass stopper to prevent the development of pressure. The contents were cooled and absorbance measured at 540 mμ.

Statistical analysis of the data revealed significant reduction in total phenolic content of susceptible variety of cotton under the stress of bacterial blight infection when compared with healthy tissues of the same variety (Fig. 1). Quantitative differences in total phenols of resistant and susceptible varieties were not observed to be significant. However, this indicated that the bacterial pathogen was able to catabolize the phenols. Leucoanthocyanins were, on the other hand, present in much larger quantities in resistant variety than in susceptible one. It was interesting to observe that about half of the leucoanthocyanins were degraded during the bacterial pathogenesis. Among the many possible basis of disease resistance and particularly among those attributable to phenols, leucoanthocyanins may presumbly be playing a vital role in conferring resistance or susceptibility to cotton by *Xanthomonas malvacearum*. Inhibition of polygalacturonase activity of yeast and *Sclerotinia fructigena* by pear and apple leucoanthocyanins have been observed

(COLE and WOOD, 1961). Besides, NEISH (1960) reported that oxidized leucoanthocyanidin inactivated both polygalacturonase and methylesterase activity and was responsible for resistance in apple. However, leucoanthocyanin-based mechanism have not been reported earlier with regard to bacterial infections.

Further studies are in progress for better understanding of the role of phenolics in bacterial blight infection in cotton.

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Sucking Trap for Observing the Swarming of Males of San José Scale, *Quadraspidiotus perniciosus* Comst. *(Homoptera, Coccoidea)*

By

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In 1970—1973 a simple, reliable sucking trap has been developed for observing the swarming of males of San José scale. The sucking trap was constructed in two modifications; in one type a ventillator blows air onto a plate coated with vaseline, which can be changed according to the necessity. In the other type the vaseline-coated plate is fastened on the rotating drum of a clockwork, which makes possible an uninterrupted, automatic sampling for a one-week period.

The sucking trap catches corresponded to the data of white, vaseline-coated plastic sheets used as control. The trap caught males even in a distance of 11 m from the trees. Besides the San José scale males, the trap collected a considerable amount of scale parasites as well as aphids, mites and *Thysanoptera*.

The use of sucking traps became widely introduced in the field entomology for collecting flying or airborne members of the insect fauna (reviewed by CHAUVIN, 1967; SOUTHWOOD, 1966). Even since the publication of the comprehensive works mentioned, many papers dealt with the use of different types of sucking traps for collecting and sampling different groups of insects: DEAN and LUURING (1970) and ELLIOTT (1971) for collecting flying aphids, FOSTER (1971) and LEWIS (1965) for collecting flies, MUELLER and ULFSTRAND (1970) for sampling caddisflies, ROTH and COUTURIER (1966) for collecting *Hymenoptera*, TAYLOR and FRENCH (1973), VAN ARK and PIENAAR (1970) for general sampling etc. The original sucking trap types have been modified to facilitate automatic samplings per hour (JOHNSON, 1950; SIMPSON and BERRY, 1973).

Surveys were carried out also in Hungary by using a sucking trap (JERMY 1969); the *Thysanoptera* material collected was determined by JENSER (1973), the aphids collected are being determined (EL KADY, SÁRINGER, unpublished data).

Material and Methods

The signalisation of San José scale is based to a considerable extent on the observation of swarming of winged males; for the latter purpose the white colour traps (plastic sheets) were found the most suitable (KOZÁR, 1972). The catches

of colour traps depend however very much on climatic and other factors, besides, the data are not significant in case of a low population density. The use of sucking traps makes possible also to study the flight of scale parasites, simultaneously with the flight of scale males.

The studies have been commenced in 1970, by using the battery-operated spore trap used in phytopathological research (VARGA, 1967); it could be attributed to the weak sucking power that this type of trap was unable to catch scale males. In 1972 we constructed a sucking trap by using a 45 W table ventillator.

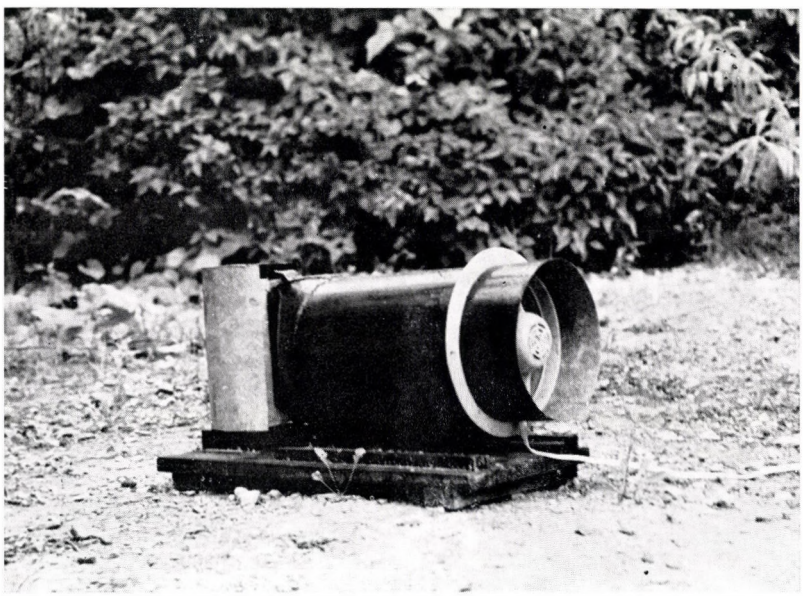

Fig. 1. Sucking trap combined with a clockwork (Photo: Kozár)

This type operated with success and caught in the period 17—24 July 1972 1413 males. The sucking trap shown in Fig. 1 was constructed in 1973, by using only a 22 W ventillator (capacity: 350 m^3 per hour). The electric fan drives the air onto the vaseline-coated sheet of the equipment, fastened on the rotary drum of a recording unit (originally used for automatic temperature recording) and the small insects sucked in with the air stream become stuck in the vaseline. The drum is protected from other contaminants (flying debris, dust) by a metal cylinder. This type is operated with 220 V main current. The trap was placed in Csopak (Balaton region, West Hungary) under low-trunk Golden Parmen apple trees. We constructed also an other type of the sucking trap where instead of the rotary cylinder a vaseline-coated glass plate is placed into the air stream in a suitable frame; the glass plate can be changed in the required intervals (hours, days, weeks).

The trap described operated for 5 months continually, without disturbances.

Results

The flight of San José males has been observed during three swarming periods. By comparing the data with the ones of the colour traps it can be concluded that both methods are useful in following the flight process (Fig. 2).

According to the studies in 1973, this type of sucking trap is suitable also to observe the swarming intensity of scale parasites. The flight of parasites commenced towards the end of the male swarming. The differences in the flight period need further investigations to decide, whether eventual control measures applied against the males may be harmful against the parasites. The majority of the parasites collected belonged to the species *Prospaltella perniciosi* (Tow.) and *Aphytis proclia* (Walk.) *(Chalcidoidea)* (Fig. 3). The flight of the first generation of males continued for 19 days, while the swarming of parasites lasted from mid-May until the second half of July with two peaks (in May and in July). The swarming of the second generation males had begun by 7 July and lasted until the second half of October.

The sucking trap used collected also to a smaller extent mites, aphids, thrips adults and larvae of San José and other scales (L_1 stage).

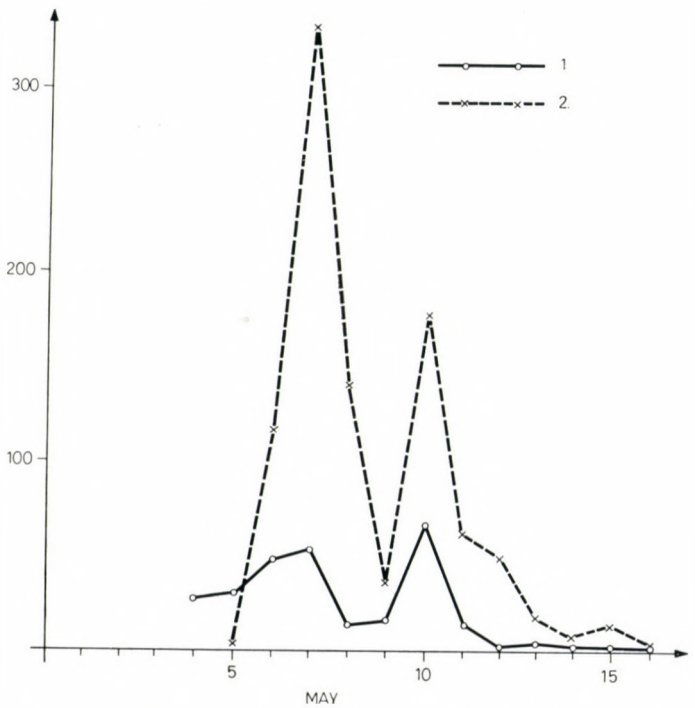

Fig. 2. Data on the male flight of San José scale, by using two different methods, on the same tree (Csopak, 1973). 1 = sucking trap, 2 = color trap, ordinate: number of males, abscissa: days (May)

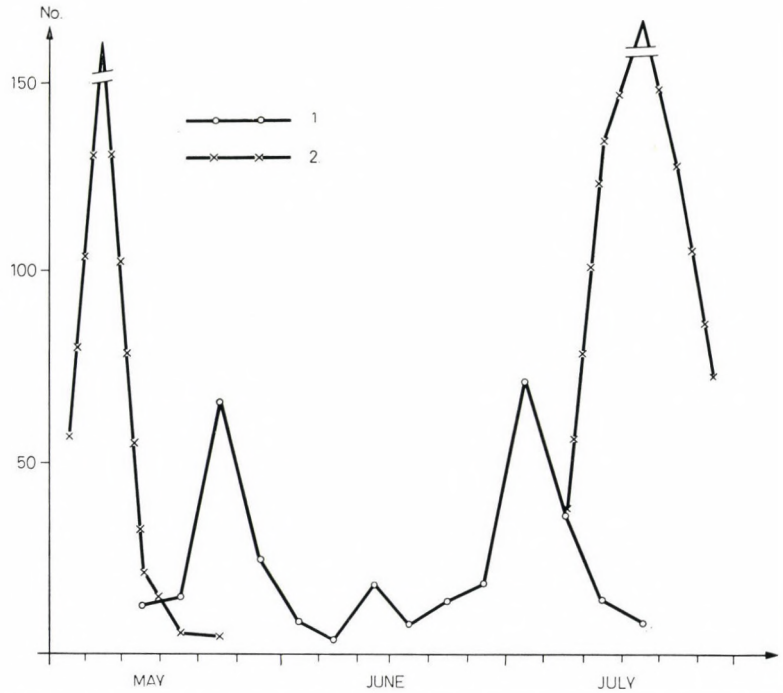

Fig. 3. Swarming of San José males and parasites in 1973. 1 = parasites, 2 = San José males, ordinate: number of males and parasites, abscissa: months (from May to July)

We studied also the occurrence of flying males in the vicinity of apple trees. The trap collected in 21 days 203 males in 1972, in a distance of 2 meters; in 1973 in a distance of 11 m from the tree 5 males were caught in 15 days, compared to the trap under the tree crown which caught 46 males during the same period. No males were caught in a distance of 22 m.

Conclusions

The sucking trap described is useful to observe the flight of San José scales and scale parasites. Its use may be recommended to forecasting stations, where electric current is available, as the operation is more difficult by using batteries (accumulators). It is advisable to place the trap in a level of tree crowns.

In orchards with no electrical source, the use of white sticky traps is recommended for observing the male swarming. For the purpose of forecasting also the modifications without clockwork are suitable.

Acknowledgements

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Inhibition of Oviposition in the Bean Weevil (*Acanthoscelides obtectus* Say, Col., *Bruchidae*)

By

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Bean weevil females laid, in choice tests, significantly more eggs on untreated dry beans as compared to those containing 0.234 mg and more $CuSO_4 \cdot 5\ H_2O$ per cm^2 of seed surface.

Keeping the weevils on dry beans treated with a 0.2 *M* solution of $CuSO_4$ caused: (a) reduction of fecundity (most probably by decreasing ovogenesis), (b) to a smaller extent, decrease of egg-viability, and (c) prolongation of the duration of oviposition period.

Thus the chemical qualities of the dry beans evoking oviposition in the weevil can be masked by $CuSO_4$.

The results indicate that the rate of multiplication can be reduced in this insect by oviposition inhibitors.

Many plants contain secondary substances inhibiting oviposition in phytophagous insects (GUPTA and THORSTEINSON, 1960; YAMAMOTO and FRAENKEL, 1960; BYRNE *et al.*, 1967; HSIAO and FRAENKEL, 1968; etc.). JERMY (1972) investigating the oviposition behaviour of the pea weevil (*Bruchus pisorum* L.) found that the females were not willing to lay eggs on the surface of pea-leaves touching the pea-pods, not even if the leaves were folded into the shape of a pod. However, they did oviposit close to the pea-pods on a glass surface providing indifferent tactile stimuli. Therefore, it could be supposed that the pea-leaves contained a substance inhibiting oviposition.

Selection of the oviposition site is based, similarly to food specialization, on a "two-way specialization" of the chemoreceptors (JERMY, 1965), thus, both stimulatory and inhibitory stimuli determine the behaviour of egg-laying females. This is obvious in the case of species the ovipositing females of which choose the food for the larvae by laying the eggs directly on or into the suitable substrate.

In the bean weevil, olfactory cues acting in a short range (POUZAT, 1974) and/or contact chemical stimuli play a decisive role in the selection of the food plant. First of all the receptors of the maxillary palpi but also those of the ovipositor participate in chemoreception. Tactile stimuli represented by the substrate,

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e.g. shape (spherical surface and gap on a curved surface) perceived by the antennae and by the proprioceptors in the legs, are of secondary importance (SZENTESI, 1976).

Egg-laying can take place, though to a smaller extent, also on other leguminous plant seeds which do not provide optimal food for the larvae, as well as on substrates having the required shape and lacking of inhibitory substances, but absolutely unsuitable for larval feeding, for example on glass beads (SANDNER and PANKANIN, 1973; SZENTESI, 1976).

The chemical stimuli inducing oviposition in the bean weevil can be masked by applying various chemicals, e.g. bordeaux mixture, 2,4,6-trichlorophenoxy acetic acid, 2,4,6-trichlorophenoxy ethanol (JERMY, 1972) on the dry beans.

The present paper deals with experiments carried out applying copper sulphate for masking oviposition stimuli originating from the dry beans. The aim was to answer the question whether egg maturation, fecundity and fertility of bean weevil females were affected by the inhibitory stimuli.

Material and Methods

Virgin bean weevil adults were gained from a laboratory mass rearing (SZENTESI, 1972) by sieving just emerging adults in half an hour intervals.

Large white beans served as oviposition stimulus, and $CuSO_4 \cdot 5H_2O$ as inhibitory stimulus. 100 g dry beans (244 $\pm$ 4.5 seeds) were thoroughly mixed in a shallow glass dish with 2 ml of a solution containing 0.05, 0.1, 0.15 or 0.2 *M* $CuSO_4$, respectively, then dried in a hot (about 80°C) air strea munder continuous shaking. The amount of $CuSO_4$ remaining in the glass dish was measured. By this means the seeds were uniformly covered by $CuSO_4$, the amount of which averaged 0.397 mg/seed or 0.234 mg/cm^2 of seed surface when treated with a 0.2 *M* solution. Dry beans treated in the same way with distilled water served as a control.

Experiment I

The dry beans were put in a one-seed layer into petri dishes of 10 cm in diameter the bottom of which was divided into four sections by a 3 mm high and 3 mm thick cross-shaped wall of paraffin wax.

The following three variants were applied:

A – In all four sections of the petri dish $CuSO_4$-treated dry beans ("total inhibition").

B – In two opposite sections of the petri dish $CuSO_4$-treated, in the other two sections water-treated dry beans.

C – In all sections water-treated dry beans.

Experiment II

One or two days old weevils were put into petri dishes containing one layer of $CuSO_4$-treated (0.2 *M*) beans and then transferred to untreated beans after 0, 3, 6, 9, 12, 15 and 18 days, respectively.

In both experiments 20 females and 20 males were put into each petri dish. Each variant was repeated 10 times. The dishes were kept at 23°C in total darkness. After the death of all females the number and viability of eggs laid, and the number of unlaid and retained eggs in the lateral oviducts were determined.

Results

Experiment I

In the variant B the overwhelming majority of the eggs (93.27 ± 13.2% in the case of 0.2 *M* $CuSO_4$) was laid on the water-treated beans, thus, copper sulphate strongly inhibited egg-laying. A hyperbolic curve was found to be characteristic for the percentage of the eggs laid on the $CuSO_4$-treated beans as a function of the concentration of the $CuSO_4$ solution used for seed treatment (Fig. 1).

The fecundity found in variant A was half of that in variant C, and differred significantly also from that in variant B ($P < 1\%$, t-test), owing to the fact that

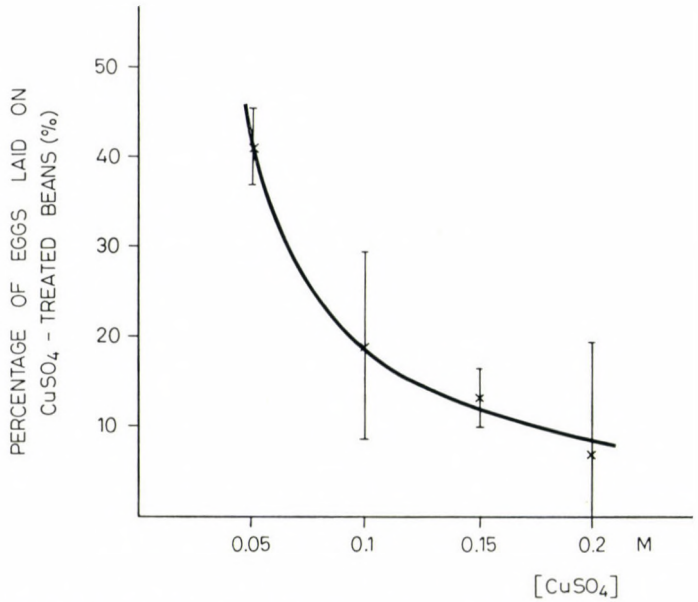

Fig. 1. The effect of the $CuSO_4$ concentrations on the percentage of eggs laid on treated beans

in the latter variant the females could choose between treated and untreated beans (Table 1).

Table 1

Effect of the presence of 0.234 mg/cm² $CuSO_4 \cdot 5H_2O$ on the surface of dry beans upon the fecundity and fertility of bean weevil females (10 replicates)

Variants	Fecundity, eggs/female	Percentage of non-viable eggs	No. of eggs retained in the lateral oviducts per female
A. treated beans only	10.3 ± 2.8	24.1 ± 9.3	2.44 ± 1.6
B. treated and untreated beans	16.6 ± 3.7	19.9 ± 7.1	1.36 ± 1.1
C. untreated beans only	20.7 ± 3.8	22.5 ± 8.3	0.84 ± 0.7

There was no difference in the percentage of non-viable eggs laid on treated and untreated beans, respectively, and there were only a few eggs retained in the lateral oviducts. The number of the latter showed a decreasing tendency according to the following order: treated beans > treated and untreated beans > untreated beans, although the differences were not significant (Table 1).

Experiment II

Total fecundity, i.e. the number of eggs laid both on treated and untreated beans, decreased with the time spent by the females on 0.2 *M* $CuSO_4$-treated beans, and there was a significant difference ($P < 0.1\%$) from the 12th day as compared to day 0 (Fig. 2, a). The number of eggs laid on treated beans (difference between *a* and *b* in Fig. 2) increased however, with a substantially lower rate than at optimal oviposition circumstances.

The percentage of non-viable eggs showed an increasing (but not significant) tendency till the 12th day and then remained almost at the same level (Fig. 3, columns-a). The percentage of non-viable eggs laid on treated dry beans did not change significantly (Fig. 3, columns-b). The same value increased continuously till the 15th day in the case of eggs laid after transferring the females to untreated dry beans (Fig. 3, columns-c), and there was a significant difference between the minimum and maximum values (16.7 ± 6.5 and 52.5 ± 24.3, $P < 1\%$).

The number of eggs retained in the lateral oviducts of females did not change with the duration of the inhibitory effect, it was at an equally low but not at a neglectable level (Fig. 2, c).

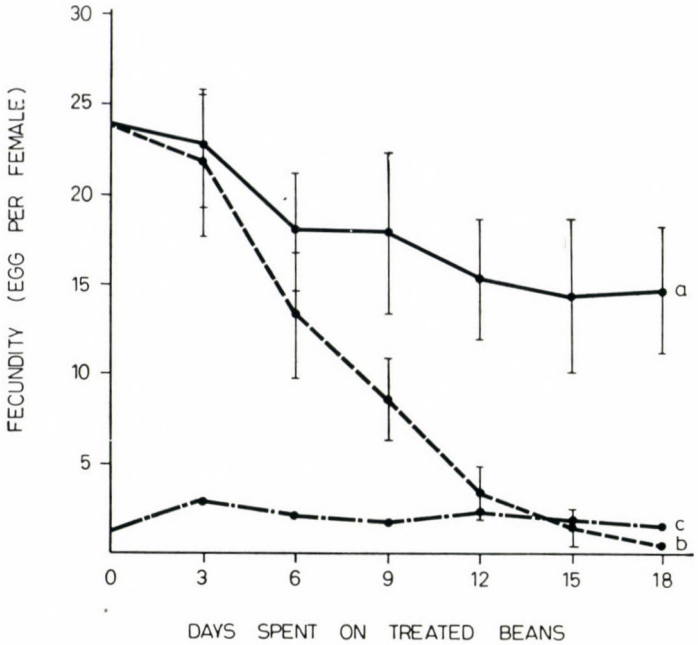

Fig. 2. The effect of duration (days) of inhibition on the fecundity and the number of eggs retained in the lateral oviducts per female: a: ———— total fecundity/♀; b: — — — — fecundity/♀ from the time of transmission to the untreated beans; c: —·—·— mature eggs retained in the lateral oviducts/♀

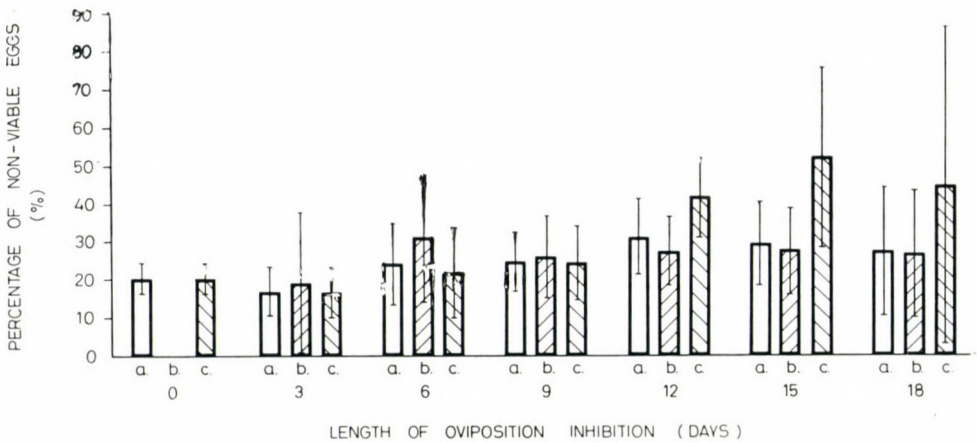

Fig. 3. The percentage of non-viable eggs: (a) laid during 18 days; (b) laid on treated dry beans; (c) laid on untreated dry beans

Discussion

Comparing *Exp. I* and *II*, there was no difference in the fecundity and fertility of females kept continuously on untreated or for one hour on treated dry beans. The same was found when females were egg-laying on treated dry beans during their lifetime or for 18 days.

The reduction in the fecundity with increasing duration of oviposition inhibition, (Fig. 2, a) and the generally low number of eggs retained indicate that the inhibition of oviposition (through the inhibitory state evoked by the $CuSO_4$) may affect ovogenesis either by slowing it down or by the resorption of immature eggs which can occur at the ovariole level (LABEYRIE, 1960). It can be supposed also that the vitellogenesis itself might be sensitive to the inhibitory stimuli (since the fecundity did not reach the normal level after the cessation of inhibition). However, these experiments are inadequate for revealing the physiological mechanisms of the influence of inhibition.

For the increase in the percentage of non-viable eggs laid after the transfer to untreated beans (Fig. 3, columns-c) several factors may be responsible: (a) the viability of eggs laid at the final period of oviposition decreases even under normal conditions (SZENTESI, 1975), (b) the inhibition of oviposition by $CuSO_4$.

It has been mentioned that inhibition may have affected the process of egg-aying also by slowing it down. Fig. 4 shows that 50% of eggs were laid on the reated beans by the 9th day. Under normal conditions and at the same temper-

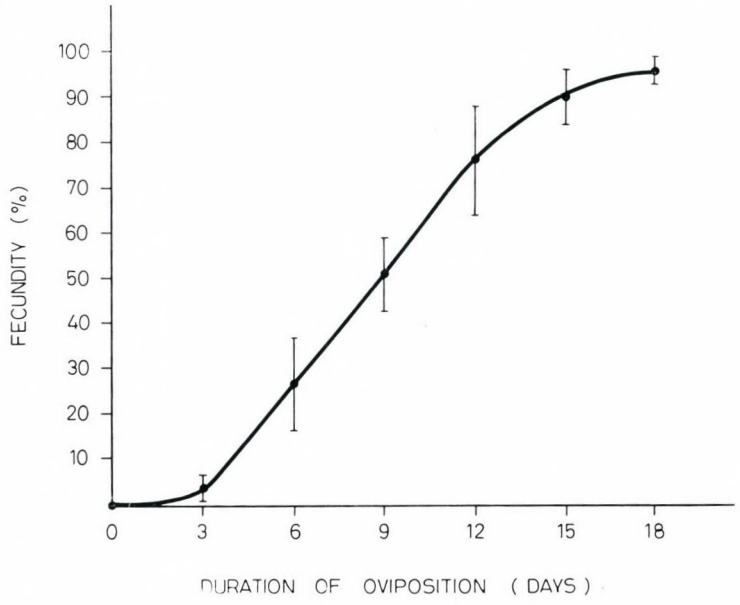

Fig. 4. Egg-laying rhythm in the presence of $CuSO_4 \cdot 5\ H_2O$

ature regime 50% egg-laying takes place by the 4th day (VUKASOVIĆ, 1949), and the same was found by SZENTESI (1972) although at higher temperature (28°C) and under crowded conditions (mass rearing).

It has been also shown by VUKASOVIĆ (1949) and SANDNER and PANKANIN (1973) that the lack of optimal egg-laying stimuli (absence of dry beans, replacing dry beans by glass beads, peas, etc.) extended considerably the beginning and duration of oviposition. Thus the presence of $CuSO_4 \cdot 5H_2O$ on the dry beans has an effect similar to the lack of optimal oviposition stimuli also regarding egg-laying rhythm.

In conclusion, $CuSO_4$ by masking optimal oviposition stimuli of dry beans caused:

(a) the decrease of fecundity,

(b) to a smaller extent, the reduction of egg viability, and

(c) the prolongation of the egg-laying period.

However, it has to be emphasized that there was no decrease in the fecundity when both treated and untreated dry beans were present, but, depending on the concentration of the $CuSO_4$ applied, the number of eggs laid on the treated dry beans proved to be significantly smaller as compared to those laid on the untreated ones.

Thus, the population dynamics of insects – at least of species having a reproduction biology similar to that of the bean weevil – can be influenced by applying an adequate quantity of oviposition inhibitors.

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Factors Determining Host-plant Selection Behaviour of Insects

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The examples studied proove that the behavioural patterns of insects related to different morpho-physiological conditions of plants play an important role in the formation of optimal feeding conditions for different groups of phytophagous insects. This ensures the species' optimal development and reproduction, the finding of most favourable feeding sites, the consumption of food and its hydrolization by the smallest energy input. At the same time the adaptation to certain morpho-physiological stages of plants helps to stabilize the utilization of food.

Among the traits of plant resistance to insects there is a basic group of factors affecting host-plant selections, which is based on the insects' behavioural reactions developed in evolutionary processes. Factors determining host-plant acceptance and/or rejection by insects in relation to different plant varieties and species have just recently become the object of detailed studies. This is a question of special importance also in connection with investigations into the nature of host-plant specificity in various insect groups.

Research work done so far in this field showed that host-plant selection by insects is determined by most various characters of the plant, e.g. by habitual, morpho-anatomical, biochemical, etc. properties.

These factors can play a role as "immunological" barriers preventing the acceptability of the plant by the insects or they can function as attractive characters for the latter. In all cases of host-plant/insect relationships one or the other factor function as a trait determining the basic form of insect behaviour. However, as our investigations (VILKOVA, 1959, 1963a, 1963b, 1967) have shown, independently of the kinds of host-plant/insect relationships, the basic feature of insect behaviour is aimed at the optimalization of nutrition.

Since the limited space does not allow to deal with the significance of all above mentioned groups of factors determing host-plant selection, in the following only the factors related to the physiological-biochemical traits of plants will be discussed. These characters are mainly determined by the presence and quantity of the so-called "secondary plant substances", i.e. terpenes, alcaloids, glycosides etc. (FRAENKEL, 1969; BECK, 1960; KLUN and TIPTON, 1966).

Admitting the significance of the secondary plant substances in host-plant selection, we came to the conclusion, on the basis of experiments conducted with

some phytophagous insect groups, that these factors are of minor importance. The adaptation for obtaining and utilizing the food more effectively is the base of food specialization. It is determined by the behavioural characters of phytophagous insect species in the course of food selection (regarding either the whole plant or a special part of it), and it is based – in case of all species – on the optimal hydrolization of essential biopolymers, i.e. proteins, fats, carbohydrates, and a series of other important substances and on their most favourable mixture in the host-plant as well (VILKOVA, 1967, 1968, 1971; VILKOVA and KOLESNITSHENKO, 1973; VILKOVA and SHAPIRO, 1968a, 1968b, 1973a, 1973b; EKMAN and VILKOVA, 1973).

Material and Methods

Behaviour and feeding of the following species were investigated: *Oscinella frit* L., *O. pusilla* Meig., *Elachiptera cornuta* Fall., *Scaptomyza graminum* Fall., *Pegomyia betae* Pz., *Bruchus pisorum* L., and several bug species belonging to the genera *Eurygaster*, *Aelia*, *Carpocoris*, *Dolycoris*, *Trygonotylus* and *Lygus*. At the same time the kinetics of the host-plant's morpho-physiological characters during ontogenesis were also studied. Special attention was paid to the properties of different stages in the plants' developmental processes because they are characteristic of not only the tendency and direction but also of the physiological and biochemical traits of plant organogenesis (KUPERMAN, 1969).

The elements of behaviour of insect species and subspecies when selecting the host-plant or its parts, the structure of mouthparts and the characters of feeding, the structure of the digestive system, and the adaptedness of the enzyme hydrolase to various substrates, were investigated. Besides visual observations of behavioural reactions, the duration of certain behavioural reactions, the duration of certain behavioural events has also been measured. For the investigations of structural characters of the insects' mouth parts and digestive systems anatomo-hystological methods have been used.

Results

All kind of behavioural reactions in phytophagous insects are aimed at the optimalization of feeding conditions, i.e. at the creation of homeostatic circumstances while utilizing food. The highspeed physiological-biochemical processes of the developing plant induced the adaptation of many phytophagous insects to the utilization of the plants in their different developmental stages. The adaptation to the feeding on plants being in various stages of morpho-physiological development has been studied on all the phytophagous insect species listed above.

Regarding the food selection in *Oscinella* spp. and in *Pegomyia betae* strict specificity can be found in these species regarding the choice of feeding site.

According to our observations, the behaviour of *Oscinella* spp. is characterized by the specialization to the feeding on the meristematic tissues of cereals.

The females ensure the larval feeding by selecting a plant being in the early stage of ontogenesis, thus providing favourable conditions for larval feeding and development (VILKOVA, 1963a, 1963b).

Opposite behaviour has been shown in *Pegomyia betae* the whole physiological specialization of which being tuned to the choice of the most differentiated plant leaf-tissues. The optimal food is supplied for the larvae by the ovipositional preference of adults for the outer leaves of the beet plant, and by the specialized behaviour of the larvae.

It has been shown that there is no clear behavioural connection between the choice of the feeding site and a specific developmental stage of the host plant in the case of the larvae of *Elachiptera cornuta* and *Scaptomyza graminum* which feed on decaying plant tissues and fungi. Their food selection behaviour is substantially tied to a certain degradation level of plant tissues and to the development of saprophytic and parasitic microflora on them (VILKOVA, 1963a).

The pea weevil, *Bruchus pisorum*, is a highly specialized species. Its host selection behaviour is tunned to late stages of the plant's ontogenesis, i.e. to the period of flowering and pod formation. Processes of growth and the stages of development of the pea plant as well as the degree of differentiation of sclerenchymatic tissues in the wall of the pods are the most important criteria of host selection in this species (KOLESNITSHENKO, 1972).

Rigid selectivity against plants can be observed in many bug species feeding on wheat at the time of the formation of grains. This corresponds to the Xth, XIth and XIIth stages of organogenesis. However, it has been shown in all the species investigated that their feeding and development is especially related to the formation, development and maturation of wheat grains. The role of stabilizing selection resulting in different ways of utilization of wheat grains as food source can be seen very clearly in this phytophagous insect group. These characters are partly constitutional (mouthparts, digestive system, the existence and activities of various hydrolitic groups of enzymes determining the possibilities of taking up and fragmenting of biopolymers of grains of different developmental stages) and partly behavioural (overcoming of mechanical barriers, e.g. pericarp).

As for the feeding of *Eurygaster* species (Scutelleridae), they are strongly adapted to various developmental and maturation stages of wheat grains. The form of the body, the structure of the head and of the mouthparts as well as the strong chitinization and differentiation of the apical parts of the latter ones, provide good possibilities for choosing the feeding site on the spikes and at the same time for finding best living conditions on the ears of graminaceous plants. The strong stylets of *Eurygaster* species are suitable for penetrating the bracts. Thus, they are able to reach various parts of the endospermium easily.

The members of the fam. Pentatomidae damaging wheat belong to 3 different genera and 2 tribes (Aeliini and Carpocorini). The narrower host plant specialization – in contrast to other members of the tribe – is more characteristic for the *Aelia* species. It means the preference for graminaceous plants and partly for wheat. The oval body-shape and the triangular, rather stretched head are advan-

tageous characters for penetrating into the complicated structure of wheat-ear and spikelet. Their well-developed mouth parts and relatively strong stylets ensure wide possibilities for choosing suitable feeding sites on the grain. Nevertheless, the degree of adaptation to the feeding directly on grains is not so pronounced as compared to that of the *Eurygaster* species. This is indicated by several behavioural characters connected with the selection of the feeding site on the wheat grain.

Bugs of the Carpocorini tribe (*Carpocoris fuscispinus* Boh. and *Dolycoris baccarum* L.) prefer wheat to a lower extent. Both species (and especially *D. baccarum*) prefer wheat only during a very short period of organogenesis and are specialized to the early stage of grain-maturation (mainly till the mealy-ripe stage). Entering the next, waxy-ripe stage of maturation the availability of the grain greatly decreases. This is related to the comparatively weak chitinization of the stylets, to the poorer differentiation of the apical part of the latter, and to the lower activity of the hydrolase enzymes. It has to be remarked that of the two species *D. baccarum* has the less appropriate mouthparts for feeding on wheat grain (KRAYNOV, 1972). The limitations in the feeding of *C. fuscispinus* and *D. baccarum* are expressed not only by the migration from the wheat fields of waxy-ripe stage to other places, but by some other characters of the feeding process, too. Before feeding they are seeking a long while for a suitable place to penetrate into the tissues and they feed on the side part of the grain where it is less protected by the bracts.

The bugs of the family Miridae show a special situation in the interactions with the wheat. On the contrary to the formerly mentioned bug species their mouthparts are not adequate to overcome hard mechanical barriers, that is to thrust through the bracts covering the grains. This fact basically determines their feeding site selection behaviour. Their optimal feeding conditions are restricted to the short period of grain-formation and only to some parts of the grain.

The best conditions for the consumption of wheat grains by the above mentioned bug species are assured at the end of grain-formation when the bracts are open to some extent not entirely covering the lateral parts of the endospermium or leaving them completely free.

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Growth and Organogenesis of Plants and Their Effects on the Formation of Behaviour in Phytophagous Insects

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Our investigations and the analysis of literature data have shown the important role played by growth and organogenesis of plants in the determination of the character of interactions between phytophagous insects and their host-plants. The stabilization of the phytophagous insects' living conditions, especially in the case of species living inside the plants, leads to the necessity of overcoming the "growth barriers" during evolution. In this connection we attach great importance to the behavioural characters of insects often being decisive for such processes. It is an important task to increase our knowledge in this field to help the development of theory and practice of plant protection.

The study of the feature of interactions between the development of insects and the development and growth of their host-plants, helps to enlighten important life-processes, which are connected with the adaptation to an even more effective consumption of the host-plant (VILKOVA, 1963). In this regard, the evolution has influenced not only the morphological characters of phytophagous insects with various life habits belonging to the most different taxonomic groups (SHAPIRO, 1963), but also factors determining their instincts and behaviour.

The behavioural characters of pollinating insects have long been in the focus of several authors' investigations resulting in a number of publications (GREENFELD, 1962). As for the phytophagous insects adapted to the consumption of different plants parts, these interactions have been studied far less. The study of behavioural aspects of phytophagous insects is of great importance in establishing profilactic protection of cultivated plants against them, and especially in developing methods for studying the nature of host-plant resistance to insect pests.

In this connection, the insects developing inside the plant are of great interest, since the plant tissues serve not only as a food source for them, but also as a special environment. The formation of homeostatic living conditions, among others the maximal reduction of harmful effects caused by kinetic processes taking place in the growing plant, is of utmost importance for the insects.

The results of investigations carried out by our institute on the living conditions of many insects living in developing plants, as well as the literature surveyed, helped to understand the formation of many adaptive behavioural characters which enable the insects to occupy such difficult niches.

These behavioural characters have appeared first of all in the imaginal stage when choosing the site and form of oviposition. It has to be taken into consideration that the ways of egg-laying and the selection of suitable plant parts for it, are factors decisively determining the progeny's living conditions. This fact can be observed especially well in larvae which are unable not only to migrate from one host-plant to an other, but even to move within different organs of the same plant (KAZANSKIY, 1935; VILKOVA, 1963).

Material and Methods

One of the most important questions of investigating host-plant/insect relationships in phytophagous insects is the insect's reaction to growth and organogenesis of the plant which are two main characters of the plant organism.

This principle is widely applied also by scientists working on plant resistance to insects in the Entomology Section of the All-Union Research Institute for Plant Protection. In order to study the traits of growth and organogenesis in plant species and subspecies, methods for enlightening the morpho-physiological alterations of the plants are widely used (KUPERMAN, 1955, 1969; SHAPIRO, 1962; VILKOVA, 1963, etc.).

The present paper deals with experimental results characterizing behavioural patterns related to oviposition and to living inside the plant, in the case of fly species showing different degrees of specialization.

Besides visual observations, special attention has been paid to investigations on the character of leaf-growth of various cereals and of the sugar beet during various stages of the plants' ontogenesis. The size of the leaves was measured daily in order to determine the duration and speed of leaf growth. Detailed observations were carried out on the oviposition to any parts of the plant and to leaves having different positions. At the same time the fate of the deposited eggs, their remaining on the plant during embryogenesis, and the mode of their shift resulting from the growth of the leaves, were also studied. Great attention was paid to reveal the living conditions of the larvae inside the plant. In connection with these studies the method of comparative analysis of the subsequent stages of organogenesis in different cereal species during the period of oviposition and of larval penetration to the growing point, was used (VILKOVA, 1960; SHAPIRO and VILKOVA, 1968).

Results

The detailed study of the behavioural characters of *Oscinella frit* L., *O. pusilla* Meig., and *Pegomyia hyosciami* Panz. as well as the growth properties of cereal and sugar beet leaves have convincingly proved that many insects are not able to lay eggs on relatively fast growing leaves and other plant parts. This can be explain-

ed by the females' inability to anchor themselves and keep the position of the body for a comparatively longer time, i.e. during the act of oviposition which takes, e.g., in the case of fritfly 40 minutes or more. Thus, these insects have to choose plant-parts and leaves which finished or slowed down development (coleoptile and the first and second leaves of the cereals). It has to be emphasized that the realization of this principle is species-specific and aroused by the differences in food-specialization of the insect. The oviposition behaviour of *Oscinella* spp. related to the choice of the suitable leaves, can be basically distinguished from those of other flies according to the above-mentioned characters.

Let us first examine the behaviour of *Oscinella* spp. which are specialized to the consumption of the growing point and the surrounding tissues of cereals. The females of *O. frit* and *O. pusilla* oviposit on and behind the coleoptile, on the sheath and the lamina of first and second leaves which cease to grow on the first days. They lay eggs also on the soil, near the seedlings of the cereals. By this means the site of oviposition and the place of favourable feeding conditions are brought close to each other (VILKOVA, 1963; SHAPIRO and VILKOVA, 1963; SHAPIRO, 1964). Our investigations showed that the preference of *Oscinella* spp. to the early developmental stages of cereals are different according to the plant species. It must be in connection with the instincts of the females by which they try to choose the most suitable conditions for the larval development inside the plant. The larvae of *Oscinella* spp. can normally develop in the stems of the seedlings of corn and cereals (wheat, barley, oat and rye) and the essential conditions for their life are ensured in the earliest growing and developmental stages. On the contrary, in many graminaceous species there is no possibility for the development of larvae because the stems are too thin in the early developmental stages (SHAPIRO, 1964).

It was found that the basic reason for different oviposition preferences of females to various cereal species altering in their dinamy and in time, was the room in the stem available for larval development. Egg-laying begins on corn already when the seedlings are partly still in the soil. Till the appearance of the 3rd leaf 80 per cent of the eggs are laid, then the number of eggs deposited sharply decreases. On wheat, as in the case of other graminaceous plants having thin stems, the oviposition period is postponed to a subsequent stage of the plant's development, i.e. to the 2–3-leaf-stage. The stem-diameter of *Phleum* sp. seedlings – regarded as a typical graminaceous plant – is small in the earliest developmental stage and the oviposition begins only 20 days after emergence but increases a lot in the 3–4-leaf-stage. Thus, the most suitable conditions for oviposition appear at different developmental phases in different cereal species, being in connection with their inner structure. So the instincts of *Oscinella* females ensure the optimization of living conditions for the larvae in various species of the graminaceous plants characterized by specific growth and organogenic processes.

In the case of the species of Chloropid flies and of other families of flies with larger body size, the contradiction between the body size and the stem diameter of cereals in the early growing stage is solved in an other way. E.g. the

larvae of *Chlorops pumilionis* Bjerk. after boring into the young shoots of cereals produce an excretion making the meristematic tissues and leaf-base to grow and become significantly thicker.

The larvae of *Pegomyia hyosciami* are specialized to the consumption of older leaves. The females lay eggs on the large fullgrown outer leaves of the sugar beet (CVETAEVA, 1964; SHAPIRO, 1964).

Growth and organogenesis of plants can be regarded as "growth barriers" playing an important role in the evolution of phytophagous insects. These "growth barriers" are overcome by the evolution of complex instincts in the insects. The instincts involve a lot of behavioural elements gained subsequently in several insect species and groups. One of the most simple elements is the sticking of eggs on the plant surface by a glue secreted by the accessory glands of the females' reproductive system. In many bug species, among others in the family of Miridae, the oviposition is a laborious task. The females stick the ovipositor into the young stems and place their eggs into the plant. The same way of egg-laying is used by the Cephids. It is well known that several leaf beetles (Chrysomelidae) gnaw small cavities into the leaf tissue and lay their eggs into them, thus ensuring optimal feeding place for the larvae. The female of *Pheadon cochleariae* F. gnaws a cavity just before oviposition, fills it with some sticky material and lays the egg into the excreted fluid (BOGDANOV-KAT'KOV, 1933).

More complex behavioural instincts are characteristic of many weevils *(Curculionidae)*. *Apion* spp. gnaw holes for oviposition. Much energy is used by them during this process for choosing the suitable site in order not to lay eggs into sclerotized plant tissues but into parenchimal tissues (KOKORIN, 1973). The leaf-roller females' "works" aim at the overcoming of "growth barriers" in order to provide the most favourable conditions for the larvae.

Behavioural reactions of these kinds are especially clear in the case of *Rhynchites bacchus* L. (KAZANSKIY, 1935): the females eliminate the negative effect of growth and organogenesis by partly gnowing through the stem of the fast developing fruits in order to ensure a required "milieu" for larval development inside the apple-fruit.

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A Comparative Study on the Effect of Diet on *Spodoptera exigua* Hb.

By

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Studies on the effect of ten larval diets and four adult diets together with starved moths on the durations of the developmental stages, pupal size, pupal weight, fecundity, adult longevity, oviposition periods and lifecycle of *S. exigua* were carried out under laboratory conditions of 28 °C & 67% R. H. The present results showed that the larval development was accelerated on sowbane leaves (9.25±0.10 days) and somewhat retarded on egg plant 12.15±0.27 days). The larval diet proved to have a slight effect on the pupal duration (range 5.60±0.11—6.95±0.11).

The lesser cotton worm *Spodoptera exigua* Hb. has been a major pest of vegetable crops and ornamental plants in Egypt.

Many authors (Taylor, 1931; Wilson, 1932; Willcocks and Bahgat, 1937; Atkins, 1960; Abd-El-makarem, 1969 and Hassanein *et al.*, 1971) have studied the biology of the lesser cotton worm in relation to host plants. But owing to the wide range of host plants that are liable to be attacked by this pest particularly in Egypt, further studies along the same line seem to be needed. In the present work, laboratory experiments were carried out on the effect of ten favourable host plants as larval diets on the biology of this pest. In addition to these diets some of the adult diets were also examined.

Materials and Technique

The leaves of the host plants; cornbine, sowbane, squash, cotton, til, okra, castor oil, tomato, egg plant and corn were compared as larval diet. The effect of each food material was studied on 20 larvae. Newly hatched larvae were reared individually on each food material in glass tubes 1 × 3 until the end of the fifth instar. Records of the duration of the larval and pupal stages were taken. The resulting pupae were measured and weighed singly 24 hours after pupation. They were then kept separately in Petri-dishes till the moths emerged. The emerging moths of each group were paired, and each pair was introduced into a mating and oviposition cage. The moths were affered sucrose solution at the concentration of 10% as food. Daily counts of eggs laid were made till moths died. The life spans of both sexes were determined.

In another experiment 10% honey solution, 10% sucrose solution, 5% sucrose solution and plain water were used as adult diets. Starved moths were used as check.

Individual couples of newly emerged moths were introduced into mating and oviposition cages, together with a piece of cotton-wool soaked in the diet. These pieces of cotton-wool were changed and the cages were examined daily until the moths died. The oviposition periods, fecundity of females and the life span of both sexes were determined. All the experiments were conducted under 28 °C and 67% relative humidity.

Results and Discussion

Effect of larval diet on the duration of the larval stage

The data presented in Table 1 show that the duration of the larval stage is affected by the different larval diets offered. The longest mean duration (12.15 ± 0.27 days) was recorded for larvae fed on egg plant and the shortest mean (9.25 ± 0.10 days) was recorded for those offered sowbane. However, intermediate means were obtained for the remaining diets.

Effect of larval diet on the pupal stage:

(1) Duration of the pupal stage

The pupal stage seems to be less affected by larval diet. Table 1 shows the effect of larval diet on the duration of the pupal stage. The shortest and longest periods recorded were 5.60 ± 0.11 and 6.95 ± 0.11 days for larvae fed on tomato and egg plant, respectively.

These results are in agreement with those obtained by WILSON (1932) who stated that the pupal stage lasted 6–8 days.

(2) Pupal size and weight

The data presented in Table 1 show that the pupal length, width and weight are affected by the diets taken in the larval stage. The most suitable diet for obtaining the largest and heaviest weights of pupae was castor oil. It increased the dimensions and the weight of pupae by 11.40 ± 0.28 mm for length, 3.40 ± 0.11 mm for width and 81.40 ± 3.84 mg for weight, respectively.

Table 1

Effect of larval diet on the developmental stages, pupal weight and pupal size of *S. exigua* at 28 °C & R. H.

Larval diet	Mean duration of		Mean pupal weight (mg)	Mean Pupal size (mm)	
	Larva	Pupa		Length	Width
Gornbine	10.50 ± 0.11	6.40 ± 0.07	49.50 ± 3.16	10.15 ± 0.21	3.05 ± 0.12
Sowbane	9.25 ± 0.10	5.75 ± 0.08	58.90 ± 1.67	10.20 ± 0.30	3.15 ± 0.18
Squash	10.75 ± 0.33	6.25 ± 0.07	59.50 ± 1.87	10.00 ± 0.21	2.97 ± 0.05
Cotton	10.00 ± 0.36	6.45 ± 0.08	55.40 ± 1.64	10.80 ± 0.22	2.97 ± 0.08
Til	12.05 ± 0.21	6.60 ± 0.09	54.00 ± 4.30	11.00 ± 0.25	2.95 ± 0.07
Okra	9.30 ± 0.15	6.75 ± 0.66	47.80 ± 1.99	10.40 ± 0.28	2.52 ± 0.42
Castor Oil	9.45 ± 0.25	5.70 ± 0.07	81.40 ± 3.84	11.40 ± 0.29	3.40 ± 0.11
Tomato	11.05 ± 0.51	5.60 ± 0.11	55.70 ± 2.38	10.00 ± 0.27	2.85 ± 0.07
Egg plant	12.15 ± 0.27	6.95 ± 0.11	46.40 ± 2.51	9.75 ± 0.29	2.67 ± 0.09
Corn	10.15 ± 0.30	5.60 ± 0.15	46.80 ± 1.83	9.50 ± 0.27	3.00 ± 0.09

Effect of larval diet on the adult stage

(1) The oviposition period

Oviposition periods of mated females of *S. exigus* have been found to be affected by larval diets offered (Table 2). The mean duration of each of the pre-oviposition and past-oviposition periods as shown in Table 2 (1.2 ± 0.13, 1.4 ± 0.27 days) was shortest in females resulting from larvae reared on corn. The longest mean durations (3.0 ± 0.49 and 2.1 ± 0.48) were obtained when the larvae were reared on tomato and castor oil, respectively. On the other hand, the oviposition period (2.9 ± 0.53 days) was shortest in case of egg plant, while sowbane gave the longest mean duration (6.8 ± 0.51 days).

(2) The longevity of moths

As a general rule, female moths lived slightly longer than the males. This was nearly common among all larval diets (Table 2). Females resulting from larvae fed on sowbane showed the longest life span (10.6 ± 0.85 days). The shortest mean longevity (6.1 ± 0.69 days) was observed in those fed as larvae on egg plant. Similarly, males produced from larvae fed on sowbane lived longer than those fed on any of the remaining larval diets. The longest and shortest periods recorded for males were 8.5 ± 0.58 and 5.5 ± 0.50 days on sowbane and corn, respectively.

Table 2

Effect of larval diet on the oviposition periods, longevity of mated moths, fecundity of mated females and life-cycle of *S. exigus* at 28°C & 67% R. H.

Diet	Mean duration of periods (days)			Mean longevity (days)		Mean No. of eggs/female	Mean duration of life-cycle
	Pre-oviposition	Oviposition	Post-oviposition	Female	Male		
Cornbine	2.0 ± 0.47	3.7 ± 0.70	1.9 ± 0.38	7.6 ± 0.54	5.7 ± 0.60	427.4 ± 94.38	20.30 ± 0.48
Sowbane	1.9 ± 0.35	6.8 ± 0.51	1.9 ± 0.43	10.6 ± 0.85	8.5 ± 0.58	758.3 ± 167.18	20.15 ± 0.47
Squash	1.2 ± 0.20	4.7 ± 0.79	1.4 ± 0.31	7.3 ± 0.82	6.8 ± 0.88	335.0 ± 99.82	20.70 ± 0.49
Cotton	1.4 ± 0.22	5.1 ± 0.38	1.8 ± 0.47	8.3 ± 0.42	6.3 ± 0.21	398.1 ± 67.10	20.35 ± 0.45
Til	1.7 ± 0.30	3.8 ± 0.32	1.6 ± 0.37	7.1 ± 0.69	6.0 ± 0.49	319.3 ± 59.43	22.15 ± 0.94
Okra	1.2 ± 0.13	5.3 ± 0.76	1.9 ± 0.21	8.4 ± 0.79	8.1 ± 0.50	462.6 ± 135.83	20.00 ± 0.40
Castor Oil	1.3 ± 0.21	6.4 ± 0.60	2.1 ± 0.48	9.8 ± 0.39	6.7 ± 0.54	607.2 ± 94.69	18.85 ± 0.30
Tomato	3.0 ± 0.49	2.9 ± 0.57	1.5 ± 0.31	7.4 ± 0.73	6.1 ± 0.41	324.9 ± 85.20	20.65 ± 0.49
Egg plant	1.8 ± 0.36	2.9 ± 0.53	1.7 ± 0.33	6.1 ± 0.69	5.8 ± 0.84	224.1 ± 51.23	22.90 ± 0.56
Corn	1.2 ± 0.13	4.0 ± 0.47	1.4 ± 0.27	6.7 ± 0.52	5.5 ± 0.50	252.5 ± 55.65	19.55 ± 0.46

(3) Fecundity of mated female

The larval diet has also a marked effect on the fecundity of mated females. It is obvious from data in Table 2 that sowbane leaves proved to be the most favourable larval diet for egg production. Females originating from larvae fed on this diet laid the highest number of eggs (758.3 ± 167.18 eggs). A contradicting result was however obtained by HASSANEIN *et al.* (1971) who stated that females fed as larvae on cotton produced the highest number of eggs. The total egg output of females whose larvae were offered castor oil (Table 2) was higher than those offered cornbine or okra. Larvae fed on squash, cotton, til and tomato gave female moths laying an average of 335.0 ± 99.82, 398.1 ± 67.10, 319.3 ± 59.43 and 324.9 ± 85.20 eggs, respectively. The least numbers of eggs (224.1 ± 51.23 and 252.5 ± 55.65 eggs) were laid by females fed as larvae on egg plant and corn, respectively.

Effect of larval diet on the duration of life-cycle

As previously stated, the larval diet has a more or less considerable effect on the various stages of *S. exigua*. Consequently, the whole life-cycle is prolonged or shortened in accordance with the larval diet used. It reached a mean of 22.90 ± 0.56 days in case of larvae reared on egg plant, and was shortened to 18.85 ± 0.30 days when castor oil was used. The whole life-cycle in case of feeding on the remaining diets ranged between 19.55 ± 0.46 and 22.15 ± 0.94 days (Table 2). These results are slightly in agreement with those obtained by HASSANEIN *et al.* (1971).

Effect of adult diet on the adult stage

(1) Pre-oviposition period

The pre-oviposition period varied according to the type of adult's food. The longest mean (2.4 ± 0.58 days) was recorded in case of feeding on 5% sucrose and the shortest (1.4 ± 0.16 days) in case of feeding on plain water. Means of 2.3 ± 0.26, 1.9 ± 0.28 and 1.9 ± 0.28 were however obtained with 10% honey, 10% sucrose and starved moths, respectively (Table 3).

(2) Oviposition period

Adults diet has also a considerable effect on the oviposition period. The shortest mean (3.2 ± 0.33 days) was recorded in case of starved moths and the longest (6.5 ± 0.60 days) in case of feeding in 10% honey or 10% sucrose (6.2 ± 0.53 days). With 5% sucrose and plain water, the mean oviposition periods were 4.4 ± 0.58 and 4.9 ± 0.23 days, respectively (Table 3).

Table 3

Effect of adult diet on the oviposition period longevity of mated moths and fecundity of mated females of *S. exigua* at 28°C & 67% R. H.

Diet	Mean duration of periods (days)			Mean longevity (days)		Mean No. of eggs/female
	Pre-oviposition	Oviposition	Post-oviposition	Female	Male	
10% honey	2.3 ± 0.25	6.5 ± 0.60	0.9 ± 0.28	9.7 ± 0.70	6.5 ± 0.76	622.6 ± 96.60
10% Sucrose	1.9 ± 0.28	6.2 ± 0.53	1.7 ± 0.25	9.8 ± 0.80	7.7 ± 0.63	588.5 ± 33.66
5% Sucrose	2.4 ± 0.58	4.4 ± 0.58	1.2 ± 0.25	8.0 ± 0.63	5.8 ± 0.73	334.0 ± 82.78
Plain water	1.4 ± 0.16	4.9 ± 0.23	0.8 ± 0.33	7.1 ± 0.04	6.3 ± 0.47	376.6 ± 82.32
Starved	1.9 ± 0.28	3.2 ± 0.33	1.8 ± 0.47	6.9 ± 0.46	5.3 ± 0.67	225.7 ± 50.05

(3) Post-oviposition period

As shown in Table 3, the post-oviposition period is slightly affected by adult's diet. The longest mean (1.8 ± 0.47 days) was recorded in case of starved moths and the shortest (0.8 ± 0.33 days) in case of plain water. Mean post-oviposition periods of 0.9 ± 0.28, 1.7 ± 0.26 and 1.2 ± 0.25 days were obtained on feeding on 10% honey, 10% sucrose and 5% sucrose, respectively.

(4) Longevity of moths

In general, the life-span of male moths was shorter than that of females. This was nearly common with all adult's diets used. Females fed on 10% sucrose showed the longest life-span (9.8 ± 0.80 days), but starved moths gave the shortest longevity (6.9 ± 0.46 days). Similarly, males fed on 10% sucrose lived longer than those fed on any of the remaining diets. The longest and shortest means recorded for males were 7.7 ± 0.63 and 5.3 ± 0.67 days on 10% sucrose and starved moths, respectively.

(5) Fecundity of mated female

The adult's food has also a marked effect on the fecundity of the females. Female moths fed on 10% honey laid the highest mean (622.6 ± 96.60 eggs) while the lowest mean (225.7 ± 50.05 eggs) was recorded for starved moths. Moths fed on 10% sucrose, 5% sucrose and plain water laid means of 588.5 ± 33.66, 334.0 ± 82.78 and 376.6 ± 82.32 eggs, respectively (Table 3).

Analysis of variance has shown that the ten tested larval diets varied significantly in their effect on the mean larval duration, pupal duration, pupal size,

pupal weight, pre-oviposition period, oviposition period, fecundity of mated female, adult longevity and the life-cycle, but their effect on the post-oviposition period was insignificant. The adult diets varied significantly in their effect on the mean oviposition period, fecundity of mated female and female longevity, while they did not vary significantly in their effect on the pre-oviposition period, post-oviposition period and adult male longevity. Days, and pupal size (range of length $9.50 \pm 0.27 - 11.40 \pm 0.29$ mm and range of width $2.52 \pm 0.42 - 3.40 \pm 0.11$ mm); but its effect was much pronounced on the pupal weight. The heaviest pupal weight (81.40 ± 3.84 mg) was obtained from larvae fed on castor oil, the lightest (46.40 ± 2.51 mg) from those fed on egg plant. The larval and adult diets proved to have an effect on the duration of oviposition periods. The longest means were obtained for females produced from larvae fed on sowbane (6.8 ± 0.51 days) and females fed on 10% honey (6.5 ± 0.60 days). Female moths produced from larvae fed on sowbane laid the highest number of eggs (758.3 ± 167.18), but egg plant and corn produced the least number (224.1 ± 51.23 and 252.5 ± 55.65, respectively). Females fed on 10% (honey produced the highest number of eggs (622.6 ± 96.60)) compared to 225.7 ± 50.03 eggs for starved moths. Adult females lived slightly longer than males, and moths of both sexes fed as larvae on sowbane or as adults on 10% sucrose lived longer than those fed on any of the other larval of adult diets. The whole life-cycle reached a mean of 22.90 ± 0.56 days in case of larvae reared on egg plant, and dropped to 18.85 ± 0.30 days when castor oil was used as larval diet.

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Comparative Study on the Antifungal Spectra of Isodehydroacetic Acid Anilides

By

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Anilide (6) and 2',6'-diethylanilide (7) of isodehydroacetic acid were prepared and tested for antifungal activity against a great number of fungi, comparatively with the known fungicides 2-methyl-benzanilide (4) and 2-iodobenzanilide (5). 2-Iodobenzanilide (5) proved to be specifically active against *Ustilago maydis*, while its 2-methyl-analogue (6) against *Botrytis allii* and *Botrytis cinerea*. Anilides of dehydroacetic acid (6 and 7) proved to possess an overall antifungal activity similar to that of compounds 5 and 6 but no specific activity against any of the tested fungi could be observed.

Following the discovery of the antifungal action of carboxin (*1*) and oxycarboxin (*2*) by SCHMELING and KULKA (1966), a number of analogous compounds have been prepared and tested for action against plant pathogenic fungi. Molecular structure *3* was thought to be the group responsible for antifungal activity. As a result of a detailed structure-activity relationship study, POMMER and co-workers (1974) came to the conclusion that the specific antifungal action against *Basidiomycetes* is maintained also if the methyl group is replaced by halogenatom or if the double bond of function *3* formes part of an aromatic ring. These investigations led to the development of the agricultural fungicides 2-methyl-benzanilide (mebenil, *4*) and of 2-iodobenzanilide (benodanil, *5*). These derivatives are recommended as fungicides for controlling cereal rusts. Both compounds were known from the chemical literature (WANSTRAT, 1873; WACHTER, 1893). They have been shown to possess a specific activity against *Basidiomycetes*.

The characteristic group *3*, believed to be responsible for antifungal activity, is present also in the molecule of isodehydroacetanilide (*6*), a compound known from the chemical literature (WILEY *et al.*, 1954), but not tested for antifungal action. It seemed us therefore indicated to investigate the antifungal action of this compound, comparatively with compounds *4* and *5*.

Steric factors are recognized as of major importance in determining the biological potency and specificity of organic molecules. In order to investigate the influence of coplanarity of compound *6* on antifungal action, also 2',6'-diethyl-isodehydroacetanilide (*7*), a compound not known from the literature, have been prepared and included into our antifungal tests. The presence of ethyl-substituents in both ortho-positions of the anilide group prevents free rotation around the

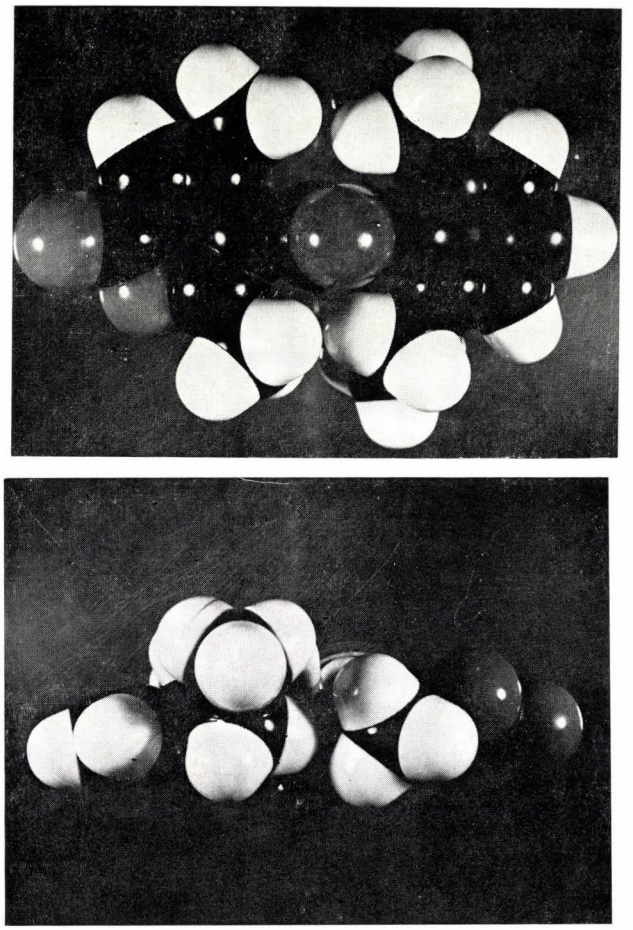


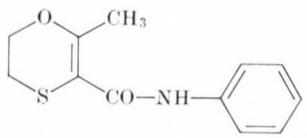
Fig. 1. Stuart-Briegleb model of 2',6'-diethyl-isodehydroacetanilide (7)

axis of the amide linkage. The Stuar – Briegleb models shown on Figure *1* indicate that free rotation is restriced by the interaction of the o-ethyl-groups with the carbonyl-oxygen of the carboxylic group.

Materials and Methods

2-Methyl-benzanilide (*4*) has been prepared according to HIRWE *et al.* (1939), 2-iodobenzanilide (*5*) according to WACHTER (1893) and isodehydroacetanilide (*6*) according to WILEY *et al.* (1954).

2',6'-Diethyl-isodehydro-acetanilide (*7*), a compound not described in literature, have been obtained on the analogy of the method described by WILEY



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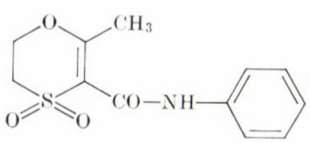
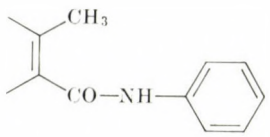


2



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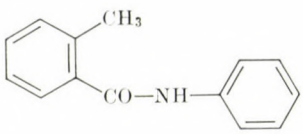

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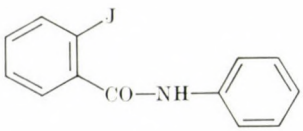

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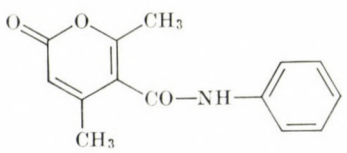

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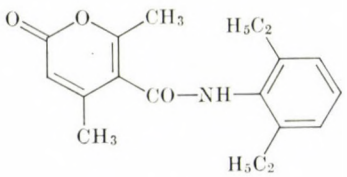

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et al. (1954), using an equivalent amount of 2,6-diethylaniline in place of aniline. Analitical and physical data are given below:

Yield 45%; mp 198–200°C (from ethanol).

Anal. Calculated for $C_{18}H_{21}NO_3$: C, 72.22; H, 7.06; N, 4.68; Found: C, 72.01; H, 7.10; N, 4.86.

Ir (KBr): νNH: 3230 cm^{-1}; νC=O (lactone): 1750 cm^{-1} (shoulder at 1715 cm^{-1}); amide-I band: 1640 cm^{-1}; amide-II band: 1515 cm^{-1}.

Nmr ($CDCl_3$): δ 1.29 ppm [*t* (7 Hz), 6 H, CH_3 (ethyl)]; δ 2.35 and δ 2.50 ppm [2 *s*, 3–3 H, CH_3 (heteroring)]; δ 2.72 ppm [*qa* (7 Hz), 4 H, CH_2]; δ 6.00 ppm [*s*, 1 H, CH]; δ 7.25 ppm [*s*, 3 H, ArH]; δ 8.1 ppm [*s*, 1 H, NH].

Table 1

Antifungal activity of compounds *4*, *5*, *6* and *7* assessed by the slide

Fungus	4	
	A	B
Phycomycetes		
Phytophthora infestans	0.24	0.27
Mucor spinosus	0.27	0.23
Basidiomycetes		
Ustilago maydis	0.02	0.022
Deuteromycetes		
Shpaeropsidales		
Cytospora cincta	0.5	0.5
Septoria apii graveolentis	0.29	0.28
Coniothyrium fuckelii	0.5	0.5
Melanconiales		
Colletotrichum atramentarium	0.11	0.26
Colletotrichum lindemuthianum	0.21	0.27
Moniliales		
Aspergillus expansum	0.5>	0.5>
Aspergillus flavus	0.23	0.2
Aspergillus niger	<0.05	<0.05
Aspergillus wentii	0.12	0.18
Penicillum expansum	0.04	0.04
Botrytis allii	<0.0005	<0.0005
Botrytis cinerea	<0.0005	<0.0005
Botrytis fabae	0.05	0.07
Trichothecium roseum	0.04	0.04
Thielaviopsis basicola	—	—
Helminthosporium sativum	0.1	0.11
Stemphylium radicinum	0.21	0.2
Fusarium culmorum	0.25	0.2
Fusarium oxysporum	0.12	0.1
Fusarium oxysporum f. cepae	0.09	0.1
Fusarium moniliforme	0.17	0.19
Fusarium solani	0.16	0.14
Micelia sterilia		
Rhizoctonia solani	0.05	0.05

Antifungal activity of the compounds was assessed by using the slide germination method recommended by the American Phytopathological Society, Committee on Standardization of Fungicidal Tests (1943), modified by BÁNKI *et al.* (1966), as well as the paper disk plate method described by THORNBERRY (1950). The filter paper disks were impregnated with a 0.5% solution of the chemical. The fungal species used as test organisms are listed in Tables 1 and 2.

germination test (ED_{50} values in concentration %)

Compound					
5		6		7	
A	B	A	B	A	B
0.22	0.25	>0.5	>0.5	>0.5	>0.5
0.36	0.37	>0.5	0.5	>0.5	0.5
0.00043	0.00036	0.2	0.31	0.05	0.035
—	—	—	—	—	—
—	—	—	—	—	—
—	—	—	—	—	—
0.29	0.31	0.29	0.26	0.17	0.16
0.38	0.25	0.5	0.5	>0.5	>0.5
<0.12	<0.12	0.25	0.25	0.12	0.12
<0.12	<0.12	0.18	0.2	0.12	0.18
0.12	0.12	0.4	0.4	<0.125	<0.125
<0.12	<0.12	0.5	0.55	<0.12	<0.12
—	—	—	—	—	—
0.1	0.15	0.34	0.42	0.19	0.39
>0.5	>0.5	>0.5	>0.5	>0.5	>0.5
>0.5	>0.5	>0.5	>0.5	>0.5	>0.5
0.1	0.12	0.44	0.29	0.5	0.47
0.35	0.37	0.25	0.21	>0.5	>0.5
0.2	0.2	—	—	—	—
0.28	0.37	>0.5	>0.5	>0.5	>0.5
0.42	0.44	>0.5	>0.5	—	—
0.29	0.3	0.37	0.38	—	—
>0.5	>0.5	0.43	0.52	>0.5	>0.5
—	—	>0.5	>0.5	>0.5	>0.5
0.5	0.5	>0.5	>0.5	>0.5	>0.5
0.25	0.25	0.37	0.4	0.5	0.5

Key: A = inhibition of germination
B = inhibition of growth

Table 2

Antifungal activity of compounds *4*, *5*, *6* and *7* assessed by the paper disk plate method

Fungus	Compounds			
	4	5	6	7
Basidiomycetes				
Coniophora cerebella	—	—	—	—
Deuteromycetes				
Melanconiales				
Colletotrichum atramentarium	+	—	—	30
Colletotrichum lindemuthianum	+	+	—	27
Micelia sterilia				
Rhizoctonia solani	+	—	—	—
Moniliales				
Aspergillus elegans	+	+	+	—
Aspergillus expansum	+	—	+	—
Aspergillus flavus	—	—	+	—
Aspergillus niger	—	—	+	—
Aspergillus wentii	—	—	+	—
Botrytis allii	+	+	—	—
Botrytis cinerea	+	+	—	30
Botrytis fabae	—	+	28	—
Fusarium culmorum	+	—	—	—
Fusarium moniliforme	+	+	+	—
Fusarium oxysporum	—	—	—	23
Fusarium oxysporum f. cepae	+	—	—	—
Fusarium solani	+	+	—	—
Helminthosporium sativum	+	+	—	—
Penicillium expansum	—	—	—	—
Stemphylium radicinum	—	—	—	—
Trichothecium roseum	—	—	—	—
Sphaeropsidales				
Coniothyrium tuckelii	+	+	—	—
Cytospora cincta	+	+	—	—
Phoma betae	—	—	—	27
Phycomycetes				
Mucor spinosus	+	+	+	—

Key: Numbers show diameter of zone of inhibition in mm; + means inhibition on the paper disk only; — means no inhibition

Results and Discussion

An overall estimation of the results obtained indicate that representatives of the anilide-type fungicides investigated in this series of experiments can be classified as fungicides of moderate general antifungal activity.

The specific activity of 2-iodobenzamilide (*5*) against *Basidiomycetes*, as published by POMMER and co-workers (1974) could be confirmed in our test. Comparison of the activity of compound *5* with that of compounds *4*, *6* and *7* against *Ustilago maydis* show that this action is highly structure-specific.

At the same time 2-methyl-benzanilide (*4*) proved to be highly active against *Botrytis allii* and *Botrytis cinerea*.

Anilides of isodehydroacetic acid (*6* and *7*) were of medium overall antifungal activity, without revealing a specific high activity against any of the fungi used as test organisms. Introduction of two ethyl-groups in *o*-positions of the anilide group, a factor resulting in prevention of free rotation, led to some increase of antifungal activity. This result indicates, that coplanarity can not be regarded as a decisive factor in the activity of this type of compounds.

The moderate antifungal activity of compounds *6* and *7* justifies the preparation and investigation of further derivatives of isodehydroacetic acid.

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Biochemical and Chemical Factors of the Selective Antifungal Effect of Triforine

II. Isomerism and chemical breakdown of triforine

By

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The technical active ingredient of triforine, a systemic fungicide shows a water solubility higher by an order of magnitude and a stronger fungicidal effectivity than the compound of analytical purity. In course of our work we rendered probable the presence of isomers, all of which are biologically active, however different from the point of view of solubility. The higher total solubility of the isomers explains the higher fungitoxicity of the technical triforine. In an aqueous solution the triforine undergoes to a chemical breakdown, limiting its period of effectivity. We studied the rate and the course of the breakdown under different conditions, by establishing which parts of the molecule may be involved in several processes.

In our earlier paper (GASZTONYI and JOSEPOVITS, 1975) we have dealt with the differences in uptake and metabolism of triforine (N,N'-bis(1-formamido-2,2,2-trichloro-ethyl)-piperazine) as causes of tolerance in case of some fungi. In course of the experiments also the chemical breakdown of the compound was discovered, which – together with the conditions of solubility – may influence considerably the biological activity. We have considered, therefore, as necessary to investigate the physical and chemical properties of triforine, the knowledge of which seemed to be necessary for a proper interpretation of its fungicidal action.

The triforine is regarded in the literature as a homogeneous compound, although the data of solubility are inconsistent. So the water solubility was reported by MARSH (1972) as 4 ppm, while the PESTICIDE MANUAL (1972), in turn, indicated 27–29 ppm. Regarding the chemical stability, a breakdown of triforine in a highly acidic or alkaline medium was observed earlier, yielding piperazine and chloroform, or chloral hydrate, respectively, as main product. The chemical breakdown in neutral, aqueous medium – the significance of which has been pointed out in our earlier work (GASZTONYI and JOSEPOVITS, 1975) – was already assumed by DRANDAREWSKI and FUCHS (1973), its rate, however, was considerably underestimated, due to lack of chemical analysis. At the same time, the photochemical breakdown of the compound was established with accuracy by BUCHENAUER (1974). Only the end product of the metabolism occurring in the plants and in the soil was presumed as piperazine (FUCHS et al., 1972; BRUCHHAUSEN and STIASNI, 1973), but also the formation of other metabolites was identified.

The differences in the biological activity of the formulated product and of the pure active ingredient were explained by some authors (FUCHS et al., 1971; DRANDAREWSKI and FUCHS, 1973) as results of the own toxicity of auxiliary agents used in the formulation; they failed, however, to give a satisfactory answer to the differences in the sizes of the active material spots, demonstrated on the chromatograms of the same materials.

In our present work we tried to contribute to the interpretation of differences in activity by comparing samples of the active ingredient of analytical purity and of technical purity, without auxiliary agents. On the other hand, we have tried to compare the pathways of chemical (hydrolytic), electrochemical (reductive) breakdown and of biological metabolism.

Materials and Methods

We have used two samples of triforine in cristalline form: the technical active ingredient (CELA W-524) and the triforine analytical standard, both supplied by the CELAMERCK Corporation. The triforine content of the technical preparation was found 96 per cent, as measured by polarography, against the analytical standard.

In the studies of some chemical properties of the two active ingredients, also the preparation Imugan (Bayer) was used, containing the active material chloraniformethane (N-(1-formamido-2,2,2-tricholoro-ethyl)-3',4'-dichloro-aniline), which belongs to the same type of compounds (from the latter no pure active material was at our disposal).

The fungicidal effectivity was studied with the method described recently (GASZTONYI and JOSEPOVITS, 1975).

In the chemical determinations (measurements of solubility, breakdown) the triforine was determined by polarography; the method used was also described in our recent work.

The polarographic analyses were completed by a lasting electrolysis with controlled potentials, to study the mechanism of electrochemical reduction. The small quantities of chloride ions produced in course of the molecule breakdown were measured with a semi-quantitative turbidimetrical analysis, by adding silver nitrate to the mixture. The presence of reactive aldehyde groups was demonstrated by the colour reaction or precipitation, respectively, produced in the presence of potassium ferri-cyanide + ferri-chloride.

For the structural comparison of triforines with technical and analytical purity, we have prepared their IR-spectrum by using a Zeiss IR-20 spectrophotometer; due to the low solubility, the materials were applied in potassium-bromide tablets. For the gas-chromatography comparisons no suitable parameters were found, because of the low vapour tension and relatively low thermostability of

triforine. In the thin-layer chromatography separations the solvent system acetonitril-acetic acid anhydrid 50 : 1 was used (developed in the CELAMERCK Corporation for the residue analysis) on HF silicagel layer.

Results and Discussion

The preparations used in the agriculture are mass-produced by all means by using triforine active ingredient of technical purity, therefore data on the biological activity utilizable in the plant protection practice can be expected only from experiments carried out with the technical active material. Regarding, however, the relations between structure and activity, only the fungicidal action of the chemically pure compound is competent. We have considered, therefore, as necessary to compare the fungicidal activity of analitically pure and the technical triforine. The action exerted on the susceptible fungus *Cladosporium cucumerinum* is shown in Table 1.

Triforine of technical purity exhibited the same fungicidal action in a concentration lower by a half order of magnitude than the material of analytical purity. To explain this difference in activity we tried to find chemical and physical differences between the two active ingredients.

Triforine decomposes by melting, the determination of the melting point is, therefore, unsuitable for purity assessments. In thin layer chromatography analysis – under the conditions indicated in the methodology – the technical active material has proved to be homogeneous and identical with the analytical standard ($R_f = 0.67$) in UV light or after developing in iodine vapour. For quantitative purposes we preferred the polarography and evaluated the wave heights of both active materials in a series of concentrations. By comparing the lowest concentrations, the technical active material proved to be of 96% purity in comparison to the analytical standard. A more essential difference was found in the concentration-

Table 1

Fungicidal activity of different concentrations of triforine (of technical and analytical purity) against *Cladosporium cucumerinum*, according to two different experimental methods

Purity grade of triforine	Impregnating concentration on agar discs			Concentration in shaken liquid culture		
	0.5%	0.1%	0.01%	0.05%	0.01%	0.005%
Analytical	+	+−	−	+	+−	+−
Technical	+	+	−	+	+	(+)

Symbols: + = complete inhibition, (+) = nearly complete inhibition, +− = partial inhibition, − = no inhibition

dependence of waves; between the concentration limits 5–150 ppm the wave heights of the technical active ingredient were proportional to the concentrations, the wave heights of the analytical standard, however, did not increase above 40 ppm and also the solution itself became turbid. This latter phenomenon indicated the lower solubility of the analytical material. Following this experiment we compared the solubilities of both materials in the media used also in the biological tests and in alcohol-water mixtures of different ratios. The determinations were carried out by polarography on the filtrates of saturated solutions, 24 hours after the onset of the estimated solubility equilibrium. The results are summarized in Table 2.

The reliability of the data was decreased by the fact that the active ingredient undergoes also a decomposition in aqueous media, therefore no complete solubility equilibrium can be attained. The equilibrium conditions were the best accessible by adding the triforine in an alcoholic solution to the water while stirring and the active ingredient content of the filtrate was determined after the surplus material produced a sediment. The precipitation was complete in one hour in the case of the analytical standard, while it took 24 hours in the case of the technical active ingredient.

The data in Table 2 exhibit a considerable difference in the solubilities of the analytical and technical active ingredients which corresponds neither to the polarography nor to the chromatography data regarding the purity of the technical preparation.

Those concentrations in which differences were established in the fungicidal activity of the two ingredients, surpassed the solubility of pure triforine. So the differences in fungicidal effectivity can be justifiably attributed to the differences in the solubility of the two purity grades. The differences in solubility are caused theoretically by two factors: a) incidental influence of impurities contained in the technical preparation, b) higher amounts of components in the technical preparation which behave identically with the analytical standard in the polarographic and chromatographic determinations.

Table 2

Concentrations of saturated solutions of technical and analytical triforine, solved in different media

Medium	Triforine of analytical purity	Triforine of technical purity
2% Ethanol	6– 8 ppm	40– 50 ppm
10% Ethanol	40 ppm	90–100 ppm
20% Ethanol	50–60 ppm	150 ppm
2% Ethanol + 2% malt	14 ppm	75–100 ppm

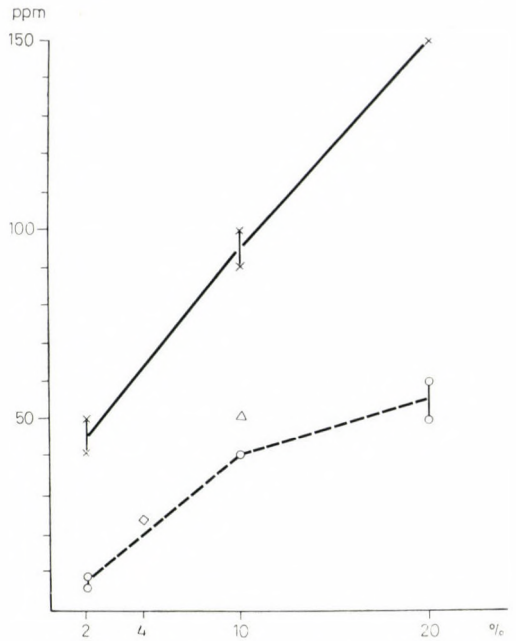

Fig. 1. Solubility conditions of triforine. Saturation curves of ——x—— = technical triforine, — — ○ — — = analytical triforine, as a function of alcohol concentration. △ = saturation concentration of the material precipitated from the solution of technical-grade triforine; ◇ = saturation concentration of the analytical standard added to the filtrate of technical triforine. Ordinate = triforine content in ppm, abscissa = concentration of alcohol

In the first case one would have expected that the precipitate of the aqueous saturated solution of the technical active ingredient would behave identically with the analytical standard from the point of view of solubility. On the other hand, the filtrate of the latter would increase the solubility of the analytical standard. The results of the experiment carried out are presented in Fig. 1, showing the solubility curves of the two active ingredients, based on the data of Table 2. Besides these curves we indicated also the saturation concentration point of the precipitate of the technical active ingredient (in 10% alcohol) and the saturation concentration of the analytical standard in the filtrate of the technical preparation (in presence of 4% alcohol). To the filtrate of the technical active ingredient we added a solution of analytical triforine in a quantity well above the saturation point (this increased the alcohol content to 4%) and determined after 24 hours with the method described above the triforine content in the filtrate of the precipitate. If the two ingredients differed only in some solubility-increasing component, then the triforine content had to be identical with the one of the saturated solution of the technical ingredient (upper curve), because the decomposed triforine quantity would be replaced from the added pure active material. The lower value obtained

showed, however, that the filtrate of the technical active ingredient did not contain any material which – similarly to the technical active ingredient – would have kept the analytical material in solution. (The rate of decrease corresponded also quantitatively to the daily decomposing amount of triforine, as it was observed also in studies which followed, e.g. Figs 4 and 5.) The solubility of the precipitate of technical triforine solution fell between the ones of the two different active ingredients, although somewhat nearer to the analytical standard. This may be explained by assuming that from among the supposed components of the technical triforine, in the precipitate the component with lower water-solubility became predominant, which corresponds to the analytical standard.

These observations indicate that from the two possible factors influencing the solubility we have to take into account the second one (i.e. the presence of more than one components). The chemical structure of triforine presents possibilities for the formation of isomers. In the formula shown in Fig. 2 the piperazine-ring is not planar, but is able to take either the chair or the boot formation [b) and c) formulas in Fig. 2]. In most amines the asymmetry of the trivalent nitrogen does not manifest itself in a demonstrable isomerism, because the enantiomer forms can transform themselves into each other via an oscillation, which demands relatively low energy input. In some nitrogen compounds with ring structure, however, the transformation is inhibited by structural causes, so the isomers can be separated (BRUCKNER, 1961). We have no data on the latter type isomerism in the case of triforine. The molecule contains in addition two asymmetric carbon atoms, which may result in further stereo-isomerism. The symmetry of the molecule decreases the number of possible isomers, on the other hand, the boot form is generally less stable from energetical point of view. Even so at least two isomers

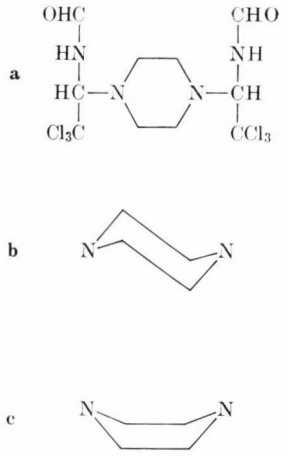

Fig. 2. a = structural formule of triforine; b = "chair" formation and c = "boot" formation of piperazine ring

may be assumed in the technical triforine, in which the relative positions of functional groups of the two side-chains are different (Fig. 3).

By comparing the infrared absorption spectra, we have looked for further evidence to prove the differences in the composition of the two active ingredients. In both spectra the absorption bands characteristic for the CCl_3-group, formamide, tertier amine and methylene groups could be identified. The unsignificant differences between the two spectra may be attributed largely to the fact that the IR spectrum prepared in the solid state depends also on the physical state and crystal structure of the material, which has been naturally different in case of our materials. We considered only one difference as essential, namely that the wide absorption band in the frequency range of $3180-3270\ cm^{-1}$ was completed in the spectrum of the technical active ingredient with a further broad band which extended to $3380\ cm^{-1}$ with a maximum in the $3320-3330\ cm^{-1}$ range. The former is characteristic for the intermolecularily associated, the latter for the intramolecularily associated NH-groups (HOLLY and SOHÁR, 1968). We interpreted this by assuming that in solid state, between the nitrogens of the formamide groups and the CO-groups of neighbouring molecules H-bridges are formed; between the two side chains of the same molecule this can take place only if the orientation of the valencies of the ring nitrogens permitted this. The two types of arrangements are shown in the formulas a) and b) of Fig. 3. The steric relationships have been controlled on an atom-calotte model. The intramolecular H-bridge increases also the polarity of the molecule by shifting the charges and this may increase the water-solubility. These together support our assumption that in the technical triforine at least two isomers are present, the one of which with an equatorial and an axial side chain (Fig. 3a), and the other with both side chains in an equatorial position (Fig. 3b). The triforine of analytical purity contains only the latter isomer, most likely because the other has been removed, due to its higher solubility, in course of the purification process. The stereo-isomerism based on the orientation of substituents on the asymmetric carbon atoms does not cause presumably alterations of this extent in the physical properties, therefore, in this regard no significant differences can be expected between the two purity grades. In course of our work we aimed by no means to separate the isomers or to produce further evidences for their presence in the technical product; we contented ourselves to establish that the technical grade triforine contained more than one, from the point of view of fungicidal action active components, the total solubility of which surpassed significantly the one of the pure (one component) active ingredient. Based on our experiments, it is very probable that the two main components differ in the relative steric orientation of the two side-chains of the piperazine-ring; this represents one case of the geometric isomerism known in tertier amines with ring structure. If both isomers possess biological activity, their higher common saturation concentration and their subsequent higher rate of uptake into the living cells present ample explanation for the significant difference in fungicidal activity observed in our experiments, in favour of the technical active ingredient. According to our opinion it is very probable that the differences in biological activity on the chromatograms

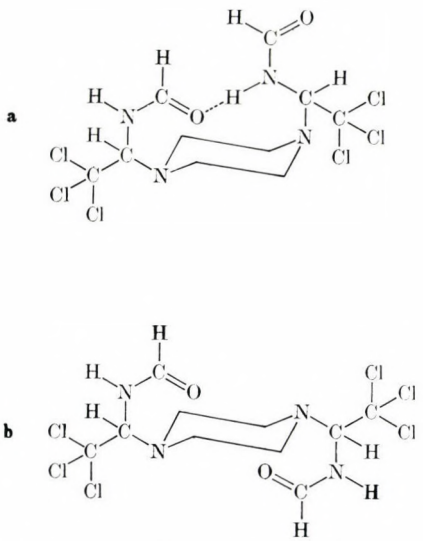

Fig. 3. Two possible steric configurations of triforine, a and b

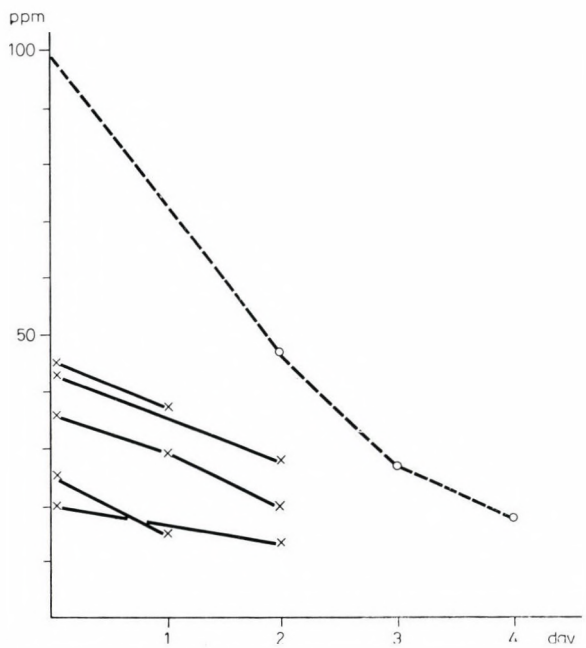

Fig. 4. Chemical breakdown of triforine at 25°C ——×—— = breakdown in 2% alcohol, ——○—— = breakdown in 2% alcohol containing also 2% malt extract; ordinate: triforine in ppm, abscissa: time in days

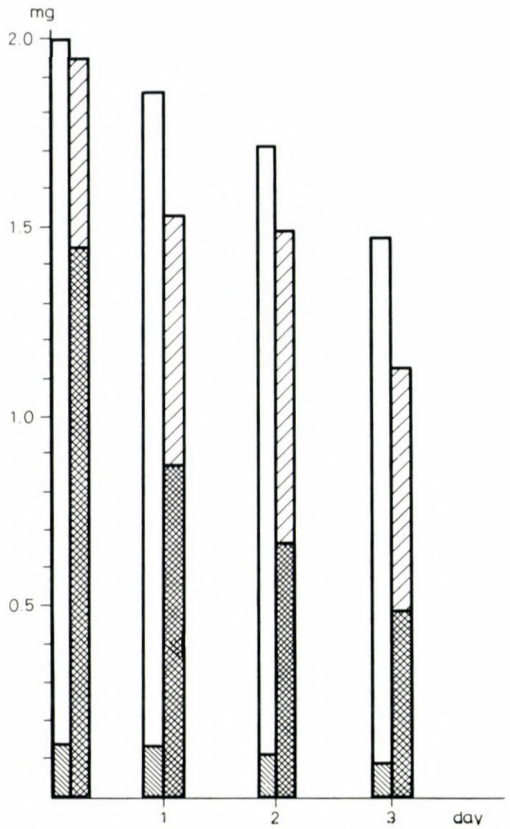

Fig. 5. Chemical breakdown of triforine of technical and analytical purity in aqueous suspension, containing 2% alcohol, at 25°C. The heights of the columns indicate the amount of triforine determined (mg) after the days indicated. ▨ = amount in the filtrate of analytical standard, □ = in the solid phase of analytical standard, ▩ = in the filtrate of technical triforine, ▩ = in the solid phase of technical triforine

of the active ingredient and of the formulated product, noted also by other authors (FUCHS et al., 1971) can be attributed to this factor. The same may be the explanation for the differences of an order of magnitude in the water-solubility data of triforine (MARSH, 1972; PESTICIDE MANUAL, 1972).

An other factor which had to be considered in the effectivity studies and which had to be quantitatively investigated, was the chemical breakdown of the active ingredient in aqueous medium. For a better comparison with the biological tests, the analyses were carried out mostly with technical triforine, in media containing 2% malt and 2% alcohol or in media containing only 2% alcohol, as used in the incubation experiments described recently (GASZTONYI and JOSEPOVITS, 1975). With both types of media the initial concentrations were saturated solutions

Table 3

Changes observed during the chemical breakdown of triforine, under different conditions, at the begin of the breakdown process

Change observed	In chemical breakdown, in neutral medium	In alkaline hydrolysis	In electrochemical reduction
Decrease of triforine	a	a	a
Decrease in fungicidal effect	f(a)	f(a)	f(a)
Electron uptake	--	—	4a
Cl^- splitting-off	2a		2a
CHO-decrease	+	+	—
Intermedier reducing at −1.2 V	+	—	—
Production of piperazine	—	+	—

Symbols: + = change occurred, — = no change occurred, f(a), 2a, 4a = change depending on triforine decrease (= a) or proportional to triforine decrease, respectively

(true solutions in the physical sense) or filtrates of 1 and 2 days old saturated solutions, respectively; the latter were regarded also as true solutions. The temporal changes in the triforine content, assessed by polarography, are presented in Fig. 4.

The breakdown of triforine could also be followed in pure 2% alcohol for a longer period, if the initial dosis was higher than the saturation concentration. In this case, however, the amounts of active ingredient had to be determined separately in the solution and in the precipitate. The data are summarized both for the technical and analytical purity grades in Fig. 5.

In Fig. 4 a remarkable difference can be noted between the breakdown rates measured in 2% alcohol and in alcohol solution containing also malt extract. In the 2% alcohol medium the rate of breakdown seemed to be independent from the triforine concentration and only in the lowest concentration (20 ppm) decreased under the average daily rate of 8 ppm. It has to be mentioned that both the malt-containing medium and the alcohol solution were of pH 5. The concentration of triforine varied in the order of magnitude of 10^{-4} M. The rate of reaction was higher in the medium containing malt and depended more on the concentration of triforine. The chemical breakdown was so significant in both types of media that the study of metabolism without considering this phenomenon would have led to wrong conclusions.

The practical conditions were better approached with the series of experiments presented in Fig. 5, in which the triforine decomposing in the solution phase was continuously replaced by the active material solved from the solid phase. In case of the analytical standard, besides the constantly low concentration in the solution, the decrease of the solid phase is well visible. The saturation equilibrium was clearly not reached in the first 4 hours in case of the technical active material

(over-saturated solution). The gradual decrease of concentration in the solution after the first day may be explained – based on the above conclusions – by the fact that simultaneously with the breakdown of the more soluble isomer, the solid phase will be enriched in the less soluble one; this will lead also to a shift in the ratio in the solution, to the advantage of the less soluble isomer.

The knowledge of the rate of chemical breakdown permitted also to make correct conclusions on the metabolism in living systems (these conclusions were already considered in our recent work mentioned above). The relatively fast rate of breakdown, however, ruled out in advance the use of chemical methods in establishing differences between the pathways of metabolism and chemical breakdown. It would not have been possible also to identify the metabolic products without using isotope labelling techniques. Instead of those we tried to find at least some footholds regarding the nature of chemical breakdown. It has been established that in a nearly neutral aqueous solution the breakdown does not follow the pathway established for extremely high or low pH ranges. While in the latter cases the first steps of the reaction seemed to represent the rate-limiting process, in the breakdown in neutral medium an enrichment of intermediers could be observed. These products can be reduced on mercury electrodes vs. n.c.e. in a range between -0.85 and -1.2 V and so these can be demonstrated on the polarogram of triforine as in this potential range the undecomposed compound does not give a reduction wave. Following a mild hydrolysis in 30% alcohol containing n/10 KOH (at 40 °C for 10 minutes), in which 46% of the initial triforine content decomposed, no intermedier of this type could be observed, nor could be other products found following the alkaline hydrolysis on the chromatogram besides the triforine residues and the produced piperazine. From the intermedier of the breakdown in neutral medium it could also be established that it was of hydrophilic character, as it remained in the aqueous phase after chloroform extraction.

Also the sequence of steps of decomposition is different in the neutral medium, compared to the hydrolysis carried out under highly acidic or alkaline conditions. The literature data (PESTICIDE MANUAL, 1972) mention as end product of the hydrolysis not only piperazine but also chloroform and chloral hydrate, respectively. In our experiments, however, in the neutral medium the production of inorganic chloride was observed, the more, in a rate which was proportional to the decrease of triforine content (the latter process followed by polarography); the amount of chloride was approximately 2 Cl/mol. Simultaneously also the amount of reactive aldehyde groups decreased. For the latter no quantitative data could be got as even the undecomposed triforine gives a substantially weaker aldehyde reaction than the compound dimethylamino-benzaldehyde used for comparison.

To complete our studies, also the circumstances of electrochemical breakdown were investigated. It has been established by using controlled potential electrolysis that the first reduction step of triforine (utilized also for determination) corresponds to an uptake of 4 electrons per molecule, i.e. to a process of twice

2 electrons. Simultaneously, by measuring the amount of inorganic chloride, the splitting-off of two Cl^- ions per molecule was established; this meant that both trichloromethyl groups are reduced by a loss of each 1 chloride ion. This latter goes parallel to the loss of fungicidal action, because in experiments in which both the triforine content and the fungicidal effectivity were studied, the two values corresponded to each other in a direct ratio. The role of the group of atoms participating in the electrochemical breakdown was elucidated from an other side by comparing the behaviour of triforine and chloraniformethane in polarographic analysis. Chloraniformethane gave with the same half-wave potential, in a twofold molar concentration the same reduction step as triforine. As the aromatic ring of chloraniformethane differs from that of triforine, this excludes the role of the ring in the electrochemical reduction. The double molar concentration was necessary, because the chloraniformethane contains in each molecule only one trichloromethyl group.

The most important changes observed in course of the breakdown which took place in a neutral or highly alkaline medium respectively, and during the electrochemical reduction (carried out at a -0.9 V potential) are summarized in Table 3.

In course of the microbiological metabolism the same changes were observed as in the chemical breakdown in neutral medium. As however the two processes cannot be separated, it is not quite sure whether all the changes observed in the latter can be associated also to microbiological metabolism.

Finally, we have to mention also some practical consequences, drawn from our experiments. *1.* If the structural conditions of a fungicidal effectivity are given in a compound, the activity can be substantially reduced by the low water solubility. In such a case the materials containing more active isomers can show a higher activity, resulting from the higher total saturation concentrations. On the analogy of this it can be expected that a synergistic effect may be reached by a simultaneous application of fungicides resembling in their action mechanism, but possessing similar low solubility. *2.* Due to its chemical lability, the residual life of triforine is rather limited; the aqueous spray liquid has to be used, therefore, in a short time of possible and it must not be mixed with other pesticides with acidic or alkaline chemical reaction. As a result of solubility and chemical breakdown, the residual life of triforine applied in plant protection operations is proportional to the amount sprayed per surface unit. The rate of breakdown, and, therefore, also the period of effectivity may be influenced by materials present in the site of application, i.e. on the surface of leaves or in the soil.

Acknowledgements

We owe sincere thanks to Mr. A. Dobrovolszky, Head of the Instrument Laboratory of the Research Institute for Plant Protection, for preparing the infrared absorption spectra described in this work.

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Biochemical and Chemical Factors of the Selective Antifungal Effect of Triforine

III. The role of the host plant in the selectivity of systemic action

By

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The causes of selectivity in fungicidal action which could be attributed to the host plant, were studied in case of the systemic fungicide triforine. In the filtrates of triforine incubated with different plant homogenates higher amounts of triforine active ingredient were found than in cases where the incubation was carried out with triforine + the pure solvent. The plant materials present in the homogenates increase, on one hand, the water solubility of triforine and decrease the chemical degradation in the aqueous medium, on the other. The homogenates of different plant species produce this effect to a different degree; the homogenates of wheat leaves showed high effectivity. It can be attributed to these factors that the systemic spectrum of effectivity does not coincide with the one of contact fungicidal effectivity. The degradation of triforine affected atom groups of the compound which are characteristic for the whole type of the related active materials, therefore the conclusions made on triforine can be — at least partly — generalized to the whole group of chloral-amide fungicides.

The authors demonstrated in a recent paper (GASZTONYI and JOSEPOVITS, 1975) that the selectivity of contact fungicidal action of triforine (N,N'-bis(1-formamido-2,2,2-trichloro-ethyl)-piperazine) can be attributed in one part of the tolerant fungus species to the lack of uptake, while in the other part the inactivation of the active ingredient takes place. However, also the role of the host plant can not be neglected in the systemic action. Some literature data already pointed out (DRANDAREVSKI and FUCHS, 1973) that there was no correlation found between the selectivity in the systemic action of triforine and the contact fungicidal spectrum of activity. As indicated by the earlier experiment, the compound (triforine) itself is active from point of view of fungitoxicity, the activation of the ingredient by the host plant could be excluded with a high probability. Data seemingly contradicting to this conclusion (DRANDAREVSKI and FUCHS, 1973), according to which the germination of conidia of mildew fungi was not inhibited by triforine applied on the leaf surface, can be attributed more to the differences in the developmental stages of the fungus itself (e.g. differences in uptake) than to the role of the host plant. The xylem transport in the plants seems to be fast and unhindered (GILPATRICK and BOURKE, 1973), independently of the plant species. By using radioactive tracer technique, FUCHS and co-workers (1972) established that triforine is metabolized in plants, they found however the metabolism similar in

different plant species. It has to be mentioned that the authors did not consider in their work the chemical degradation of the compound which has been demonstrated in our recent paper (JOSEPOVITS and GASZTONYI, 1975) and which had been rendered probable already by DRANDAREVSKI and FUCHS (1973).

We tried to contribute to the knowledge of selectivity depending on the host plant, by studying the rate of inactivation of triforine in homogenates of different plants. Similarly to microbial metabolism (GASZTONYI and JOSEPOVITS, 1975) also in this case the possibility of enzymatic metabolism had to be considered, although there were indications contradicting this (DRANDAREVSKI and FUCHS, 1973). Based on a microbial evaluation carried out following a treatment on intact plants, the latter authors suggested the possibility of a protective effect of plants against the chemical degradation of triforine, observed otherwise only by a biological test. To exclude uptake and translocation factors, we used in our experiments plant homogenates and followed quantitatively the changes in the active ingredient content, similarly to the method used in the work described in our two earlier papers in this matter.

Materials and Methods

In the experiments we used a triforine active ingredient of technical purity (96%), produced by the firm CELAMERCK.

The following plant species were used as biological objects: two-weeks old seedlings of wheat (Fertődi variety), bushbeans (Cherokee variety) in the two trifolial-leaf developmental stage, cucumbers (Marketer variety) in the two true-leaf stage.

The plant homogenates were prepared by using for each variation 1 g (wet weight) plant material; the plants were surface sterilized (by omitting the cut surface) in a 10% H_2O_2 solution for 3 minutes, washed three times with sterile distilled water, then homogenized for 5 minutes in a blendor (type MSE). The homogenates were incubated with triforine for 24 hours in shaken bottles in sterile incubation's medium. In case of wheat only the leaves were processed, while in case of beans the primary and trifolial leaves together and in cucumbers the cotyledons and true leaves together were processed. The incubation was carried out at 25–26°C, in darkness, with moderate speed on a rotary shaker type Vibroterm. The unmetabolized triforine content was determined in the filtrate of the incubations mixture by using polarography. The experiments were carried out with three replicates.

Results and Discussion

Homogenates of wheat, bean and cucumber plants were compared from point of view of influence on triforine. The experimental variations were the following for each plant:

1. 100 ppm triforine in 20 ml 2% ethyl alcohol;
2. 100 ppm triforine in 20 ml 2% ethyl alcohol + 1 g (wet weight) plant homogenate.

The triforine content measured in the filtrates after 48 hour's incubation period was expressed in the ratio of the variation not containing plant material; the results are summarized in Table 1.

Table 1

Unmetabolized triforine content of filtrates of 100 ppm triforine, incubated with different plant homogenates for 48 hours at 25°C, expressed in the % of the control without plant material

Plant species	pH of homogenate	Triforine content
—	4.9	(100%)
Wheat	5.1	165%
Bean	6.2	129%
Cucumber	6.2	122%
		$SD_{95\%}$ 25%

As it was already known from our earlier work (JOSEPOVITS and GASZTONYI, 1975) that in case of a nominal initial concentration of 100 ppm triforine the mixture contained in spite of the chemical degradation still considerable amounts of solide active material after three days, the amounts of triforine in the filtrates, surpassing the 100% of control, could be attributed either to an increase in triforine solubility or to the decrease of chemical breakdown. In any case, biological metabolism could not be demonstrated in the homogenates. The differences found in the triforine content of different plant homogenates could not be explained with the differences in the pH values, as even in case of wheat the least change was noted.

A considerable excess of triforine was observed only in case of the wheat homogenate, as the value of bean homogenate reached just the limit of significance; there was however a marked trend in each case to triforine values surpassing the ones of the control.

In the following experiments we continued the studies of wheat homogenates, which had yielded the significant differences, to establish whether the active ingredient surplus resulted from changes in the solubility or in the rate of breakdown. In separate experiments we filtrated the mixtures pre-incubated for 24 hours as described above, together with the control (the latter not containing plant material); changes in the triforine content of the filtrates were expressed in the percentage of the initial concentration (Fig. 1).

The results indicated a decrease in the breakdown rate in the presence of wheat extract.

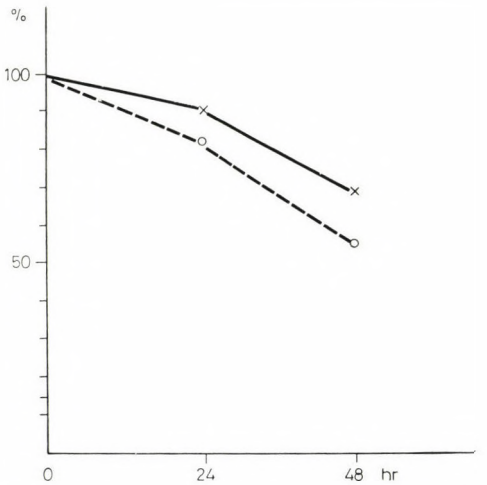

Fig. 1. Changes in the triforine content of filtrates incubated for 24 hours; ——×—— = = wheat homogenate, — — ○ — — = control without plant material. Ordinate: triforine content expressed in percentages of value determined after filtration, abscissa: time in hours

In an other experiment we studied the influence of homogenates on the water solubility of triforine. Here the homogenates of wheat leaves (1 g wet weight each) were incubated with amounts of triforine corresponding to 100 ppm in 20 ml, the latter without pre-incubation; the filtrates were compared for triforine content with the ones of the control. The triforine content of the same filtrates was determined again after 48 hours, to establish the differences in the breakdown. Data summarized in Table 2 show – beside an apparent slowing-down of the breakdown rate – significant increase in triforine solubility in the presence of wheat homogenate.

In our earlier work (JOSEPOVITS and GASZTONYI, 1975) we established a 40–50 ppm solubility of technical-grade triforine under the given experimental conditions (2% alcohol medium, at 25°C). The apparent solubility of triforine

Table 2

Unmetabolized active ingredient content of triforine filtrates incubated without and with wheat homogenate for 24 hours, determined after filtration and after 48 hours

Plant homogenate	Triforine content of filtrate		Decrease during 48 hours	
	After filtration	After 48^h	In ppm	In %
Wheat leaf	66 ppm	50 ppm	16 ppm	24%
—	44 ppm	28 ppm	16 ppm	36%

increased, therefore, in the filtrate of wheat homogenate by about 50%. There was a possibility that the remnants of leaves (plant fibers) played a role in the apparent increase of solubility, by increasing mechanically the solution rate of the solid phase; we have determined therefore also the triforine content of a filtrate incubated with quartz sand instead of leaf homogenates and observed values corresponding to the control. The increase of triforine solubility had to be attributed, consequently, to the chemical action of plant materials.

We explained the solubility-increasing effect of wheat leaf homogenates by supposing that materials released from the plant tissues produced conjugates with one part of triforine present, which are able, in turn, to go into solution even in an incubations medium saturated with free triforine. These conjugates break apart under the circumstances of the analysis (20% alcohol, pH = 9) and the triforine component becomes determined together with the free active ingredient. The conjugate is, however, supposedly more stable against the chemical breakdown in the incubations medium than the free triforine, so only the latter participates in the breakdown process. No studies have been carried out regarding the nature of the plant materials involved, nor regarding the way of linkage. It has to be mentioned that in earlier experiments with fungi (GASZTONYI and JOSEPOVITS, 1975) the malt extract used as incubations medium increased also the amount of triforine in the solution; there was, however, a faster breakdown in the presence of malt extract than in water or in 2% alcohol (JOSEPOVITS and GASZTONYI, 1975). There was seemingly a contrasting effect of wheat leaf homogenate on the stability of triforine, although the decrease in chemical breakdown could not be observed in each case. In the experiment illustrated in Fig. 1 both the percentual and the absolute decrease of triforine were lower in presence of plant materials than without. In contrast, the data of Table 2, showed in case of a higher initial triforine concentration, only a percentually slower breakdown and the decrease in triforine content expressed in ppm was identical in both variations.

We can conclude therefore, that in presence of wheat leaf homogenate (and to a smaller extent in presence of other plant homogenates) more triforine could be found in the incubations medium, than without homogenates; this active ingredient surplus could be attributed more to the increase in triforine solubility and supposedly, partly also to the decrease in the chemical breakdown. Under the circumstances of a practical application, the amount of triforine taken up by the host plant has to face a much higher amount of plant material than in our experiment; the effects observed may occur in the practice to a greater extent. Our results present an explanation for the observation of DRANDAREVSKI and FUCHS (1973) on the longer persistence of triforine taken up by the plant. We attribute however a much higher significance to the conjugates with plant materials, which increase the uptake and translocation of the active ingredient within the plant by increasing the solubility of triforine, than to the decrease of chemical breakdown. It could be also concluded from our experiments that there are differences among the plant species in the effectivity on triforine solubility and stability. This factor may influence considerably the systemic activity, explaining thus the

differences between the contact and systemic spectra of activity, established already by other authors (FUCHS and co-workers, 1971). The remarkably high systemic activity exhibited against the wheat powdery mildew may be attributed therefore not to an activation occurring within the plant tissues, but to materials present in the wheat, which increase the solubility (and thus the uptake and translocation) as well as the stability of the compound. Consequently, triforine may be applied as a systemic fungicide in the first place against the fungus diseases of those host plants, which possess these materials.

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Book Review

Ministry of Agriculture, Fisheries and Food, Plant Pathology Laboratory, Hatching Green, Harpenden, Herts.: "*Key for the Field Identification of Apterous and Alate Cereal Aphids with Photographic Illustrations*" Agricultural Development and Advisory Service publ., 1974, pp. 14.

In the last two decades much attention has been paid to aphids as the causal agents of crop losses both by sucking plant sap and by disseminating plant pathogenic viruses. Among the aphids the species occurring on cereals are of special importance and all the work related with the control, breeding for resistance and other research work calls for a great deal of field surveying. To our knowledge no key has hitherto been available for the field identification of cereal aphids, useful both for trained entomologists and non-specialists. This exquisite, pocket-size booklet offers all the advantages necessary for a field guide: it contains a short and concise determination key of aphids living on different cereals (based on characteristics discernible with the naked eye or with a pocket lens), followed by colour photographs of the aphids "in situ" on the hosts. The photographs are supplemented on the opposite pages by linear drawings indicating the most important marks of recognition. The species dealt with are: Sitobion fragariae Walker, Sitobion avenae Fabricius, Metopolophium dirhodum Walker, Metopolophium festucae Theobald, Schizaphis graminum Rondani, Rhopalosiphum padi Linnaeus, Rhopalosiphum insertum Walker, Rhopalosiphum maidis Fitch.

The pages are bound into plastic sheets, rendering the small volume durable and weather weatherproof. One of the authors, R. N. B. Prior is a well-known name in aphid taxonomy and the coloured figures are a fine work of J. R. Morrison, the Laboratory's photographic specialist.

It is to be hoped for that further field guides of similar kind will be published by the Plant Pathology Laboratory; these will be surely appreciated by virologists, entomologists and agriculturists.

L. SZALAY-MARZSÓ

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Acta Phytopathologica Academiae Scientiarum Hungaricae, Vol. 11 (3—4), pp. 149—153 (1976)

Control of Potato Late Blight with Potato-derived Phytoalexins

By

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The antifungal activity of potato-derived phytoalexins, rishitin and phytuberin, at several concentrations, were tested against *Phytophthora infestans* on leaf disks of potato. Both of the compounds significantly inhibited the growth of the fungus at the concentration of 100 ppm. Rishitin and phytuberin were applied by spraying the whole plants prior to, and after, inoculation with the same fungus. Pretreatment with rishitin at 200 ppm produced a considerable control of the late blight disease, but treatment one day after inoculation caused no visible reduction in disease symptoms. Phytuberin did not reduce the spread of disease in either case, even at the highest concentration (200 ppm) used.

Phytoalexins exhibit significant antifungal activity *in vitro* against numerous plant parasites. Furthermore, these postinfectional compounds accumulate faster and/or in larger amounts in resistant, rather than susceptible host–parasite interactions. Because of these characters, several research workers assume phytoalexins to be primary factors of plant resistance. Some findings along this line however, contradict the so-called "phytoalexin theory" and ephasise the involvement of phytoalexins in cross protection (ELLISTON *et al.*, 1971; SKIPP and DEVERALL, 1973; ÉRSEK, 1975). The paper of WARD *et al.* (1975) is the only one in which the possibility of the application of phytoalexins as fungicidal chemicals for plant disease control is discussed. The authors reported that capsidiol (a phytoalexin from pepper fruit) sprayed on tomato plants effectively inhibited the development of late blight disease at a concentration of 5×10^{-4} *M*, while at 2.5×10^{-3} *M* (approx. 500 ppm) the inhibition was virtually complete.

Of the phytoalexins obtained from potato, rishitin, phytuberin and lubimin have received the greatest attention as regards their *in vitro* antifungal activity. All of the three phytoalexins quite similarly inhibited zoospore germination of *Phytophthora infestans* [(ED_{50}: approx. 2×10^{-4} *M*) TOMIYAMA *et al.*, 1968; VARNS, 1970; METLITSKII and OZERETSKOVSKAYA, 1973)], but ED_{50}-values of rishitin, phytuberin and lubimin for mycelial growth were different, i.e. 1.5×10^{-4} *M*, 2.7×10^{-4} *M* and 6.8×10^{-4} *M*, respectively (BECZNER and ÉRSEK, 1976). The ED_{50}-values clearly indicate that rishitin and phytuberin are able to act against both zoospore germination and mycelial growth of *P. infestans* with similar efficiency.

Therefore, we chose these two latter phytoalexins for investigation of how they could serve as fungicidal chemicals on green potato leaves and plants against late blight disease.

Materials and Methods

Leaf disks 2.5 cm diam. were cut from the middle-age, fully expanded leaves of 6 to 8-week-old potato plants (cv. Desirée). Leaf disks were placed in Petri dishes containing polystyrene granula wetted by the solutions of either rishitin or phytuberin of different concentrations. After an incubation for 6 hrs at 18°C in the light, the lower surface of leaf disks were inoculated with 10^5/ml zoospore suspension of *Phytophtora infestans*, race 1.2.3.4. Inoculated disks were left in the same conditions for 12 hrs, then infection droplets were removed and leaf disks turned over. The samples were further incubated 12 hrs in the dark and 12 hrs in the light, for no more than 5 days.

Rishitin and phytuberin was obtained as described by LYON (1972). Solutions were prepared by dissolving rishitin or phytuberin in 95% ethanol and mixing this with 20 volumes of water. Control solutions contained the same proportions of ethanol in water.

For microscopic observations infected leaf pieces up to 1 cm^2 were fixed in 3 : 1 (v : v) ethanol – acetic acid until the leaves were colourless. This treatment was followed by immersion in a mixture of 95% ethanol and lactophenol (1 : 4, v : v), for 3–5 hrs. Samples were stained with lactophenol-cotton blue, and washed in lactophenol shortly before microscopic observations.

Potato plants, 6–8 weeks old grown under greenhouse conditions, were sprayed twice a day with phytoalexin solutions prepared as above. The solutions were applied either 2 days before, or one day after inoculation, by spraying with a 10^5/ml zoospore suspension of *P. infestans*. Incubation of plants was carried out for 72 hrs in a dew chamber (18°C, 12 hrs light and 12 hrs dark).

Results and Discussion

On leaf disks, both rishitin and phytuberin gave inhibition of the disease development at concentration as low as 25 ppm. On the control (untreated) disks, the size of the continuos tissue necrosis corresponded to the size of infection droplet, and visible mycelia occurred on the surface of leaves 4 days after inoculation. At concentrations of 25 and 50 ppm the size of the necrotic area of the underlaying tissues appeared to be smaller than that of infection droplet, and no surface mycelia were seen. At concentrations as high as 100 and 200 ppm the occurrence of very small and distinguishable necroses indicated the effectiveness of rishitin and phytuberin against *P. infestans* (Figs 1 and 2).

Light microscopic observations showed that 16 hrs after inoculation there was no considerable difference in hyphal length between the untreated control and the treated samples. In the case of the control, hyphae continued to grow further, but in the presence of either rishitin or phytuberin at 100 ppm hyphal growth virtually stopped (Table 1).

These results indicate that both rishitin and phytuberin are able to inhibit the development of *P. infestans*, not only *in vitro*, but also when they are applied on the plant, or at least on plant leaves. As the contact between the phytoalexins and leaf disks was continuous, more satisfactory results on the fungicidal nature of

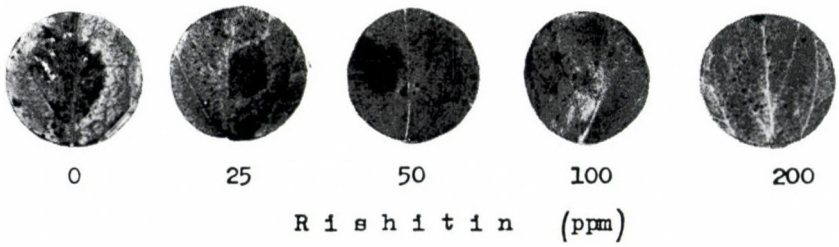

Fig. 1. Effect of rishitin at different concentrations on the growth of *Phytophthora infestans* on potato leaf disks, and on the formation of symptoms

Fig. 2. Effect of phytuberin at different concentrations on the growth of *Phytophthora infestans* on potato leaf disks, and on the formation of symptoms

Table 1

Hyphal length of *Phytophthora infestans* in potato leaf disks in the presence of rishitin or phytuberin

Phytoalexin	Hours after inoculation		
	16	32	64
Rishitin, 100 ppm	89.6± 37.5[a]	99.1± 27.5	108.0± 21.0
Phytuberin, 100 ppm	91.6± 28.4	96.4± 25.3	112.0± 18.5
Nil (Control)	104.0± 28.0	297.7± 29.5	∞[b]

[a] Means and errors for hyphal length given in μm
[b] Hyphae too long and branched to measure

phytoalexins was obtained by using whole plants for treatments and by spraying the plants with the phytoalexin.

When rishitin at 200 ppm sprayed on potato plants prior to inoculation with a zoospore suspension of *P. infestans*, significant protection was observed (Table 2). On the controlled plants only the lowest leaves exhibited some slight disease symptoms by 6 days after inoculation. In contrast, the untreated control plants were heavy infected and lost their lower leaves by this time (Fig. 3). In

Table 2

Late blight disease development[a] of potato plants controlled by rishitin or phytuberin

Phytoalexin	Time of application of phytoalexin	
	Pre-inoculation	Post-inoculation
Rishitin, 200 ppm	+	+++++
100 ppm	++++	+++++
Phytuberin, 200 ppm	+++++	+++++
Nil (Control)	+++++	+++++

[a] Degree of the disease was estimated by visual observation,
+++++: severe disease symptoms, +: slight disease symptoms

Fig. 3. Control of late blight with rishitin. Left: rishitin (200 ppm) treated, right: nontreated potato plants, 6 days after inoculation

addition, these plants died 12–14 days after inoculation. At lower concentrations rishitin gave very slight or no protection.

If the treatment was done one day after inoculation, no protection occurred. This fact seems surprising, because mycelia were more sensitive *in vitro* to rishitin than were the zoospores of *P. infestans* (WARD *et al.*, 1974). The explanation for this contradiction is that rishitin does not penetrate in sufficient quantity into the inner cells of the leaves soon enough, or hyphae have to be in contact with the compound from the beginning of hyphal growth.

Phytuberin at a concentration as high as 200 ppm (higher concentration was not attempted) did not cause any reduction in disease symptoms (Table 2), probably because of its low stability.

According to the results given here, one could not propose final suggestions for the future use of phytoalexins in chemical control of plants, because there are many problems to be solved. The chemical modification or the application of good carrier compounds would possibly promote the action of such chemicals.

The natural character and the potential antifungal activity of phytoalexins, might incite chemists to model this type compound for profitable applications in plant control.

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Acta Phytopathologica Academiae Scientiarum Hungaricae, Vol. 11 (3—4), pp. 155—160 (1976)

Effect of Rust Infection on the Cytokinin Level of Wheat Cultivars Susceptible and Resistant to *Puccinia graminis* f. sp. *tritici*

By

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Compatible and incompatible rust-race/host-cultivar combinations exhibited increased levels of cytokinin in the primary wheat leaves. The rust induced increase in cytokinin content was more intensive in the compatible combination than in the incompatible one. The same active compounds, chromatographically similar to the free base and the nucleoside form of zeatin and N^6-(Δ^2-isopentenyl)-adenine and to its riboside are present in extracts prepared from both healthy and infected seedlings of the two combinations. However, each extract contains additional active factors with R_f values different from that of the standards applied in this work.

The relationships of different growth regulators and symptoms caused by rust fungi have been investigated in several studies (Kiermeyer, 1958; Daly and Inman, 1958; Bailiss and Wilson, 1967). The endogenous cytokinins of rust infected plants, in addition to their morphogenetic significance, and their action on leaf abscission (Gáborjányi *et al.*, 1972) have an important role in the transport and accumulation of nutrients towards and around the infection courts (Bushnell and Allen, 1962; Thrower, 1965; Pozsár and Király, 1966; Király *et al.*, 1967; Dekhuijzen and Staples, 1968).

Although several studies have been carried out on this field, no effort was made to determine cytokinins in rust infected wheat, or to find correlation between the cytokinin content and resistance of wheat to rust. The present study was initiated to answer these questions. For this reason we compared the cytokinin levels of healthy and infected leaves in compatible as well as in incompatible host–parasite relationships 10 days after inoculation.

Materials and Methods

Plant material

Little Club (*Triticum compactum* C.I. 4066), and Vernal (*Triticum dicoccum*, C.I. 3686) wheat cultivars were grown in the greenhouse at about 21° C.

Pathogen

Puccinia graminis (Pers) f. sp. *tritici* race 11 was used throughout the experiments. The cultivar Little Club is susceptible to this race (3^{++}, 4^{-} reaction type) while Vernal is resistant (0; and 1 = reaction type).

Inoculation procedure

The primary leaves of 8 days-old plant were washed with tap water and sprayed with 50 mg urediospore/20 ml tap water suspension uniformly. At this concentration infection density was 60–80 pustules or lesion/leaf. After spraying, plants were placed in a humidity chamber for approximately 12 hr.

Cytokinin extraction and determination

Cytokinins were extracted from healthy and infected wheat seedlings as we described previously (SZIRÁKI and GÁBORJÁNYI, 1974; SZIRÁKI *et al.*, 1975). For further purification and separation the extracts were streaked on Whatman No. 1 filter paper and developed in isopropanol–ammonium hydroxide–water, 10 : 1 : 1 v/v or in *t*-butanol–ammonium hydroxide–water, 3 : 1 : 1 v/v. After dividing the chromatograms into 10 equal strips each strip was tested for cytokinin activity in soybean callus bioassay. For characterization of the active materials, authentic cytokinins: zeatin (Z), zeatin riboside (ZR), N^6-(Δ^2-isopentenyl)-adenine (2iP), N^6-(Δ^2-isopentenyl)-adesonine (IPA) were chromatographed in the same solvents.

Results

Twelve days after inoculation (sporulation stage) cytokinin extracts were prepared from infected and healthy Little Club seedlings. Fig. 1 shows, that the most active cell division factors in both cases can be found at R_f region 0.5–0.7. The extract of infected seedlings exerts higher total cytokinin activity (130%) than that of the healthy seedling (100%). It is also seen that the migration of these active materials is similar to that of zeatin and zeatin riboside.

In order to determine whether an increased cytokinin level is also characteristic for the resistant combination we compared the cytokinin activity of extracts prepared from healthy and diseased Vernal wheat cultivar. Samples were taken 10 days after inoculation.

In the incompatible (resistant) host-parasite combination the total cytokinin activity was about 113% compared to the cytokinin activity in the healthy control. This slight increase was found in each of the five replications. The most active cell division factors can be found at R_f region 0.5–0.8, that correspond to the migration of Z, ZR and IPA and 2iP (Fig. 2). It is noteworthy that infected extract contained an additional cell division factor located at R_f 0.2. In order to find more detail on the cytokinin composition of the extracts we used an additional

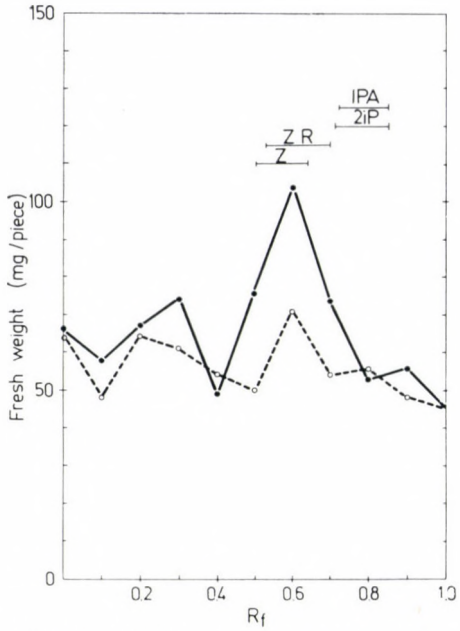

Fig. 1. Distribution of cytokinin activity on chromatograms of extracts of healthy (open circles) and infected (closed circles) plants. The chromatograms were developed in isopropanol–ammonia–water (10 : 1 : 1). The extracts were obtained from 70 g fresh leaf material/1000 ml medium

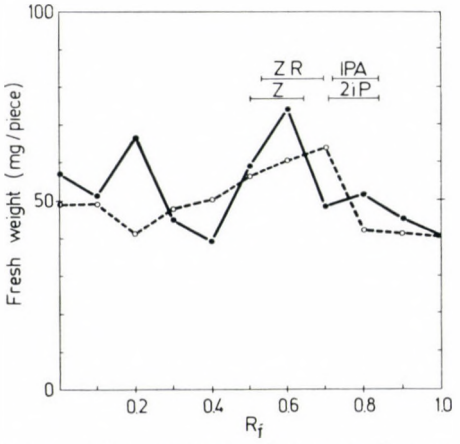

Fig. 2. The paper chromatographic separation (isopropanol–ammonia–water 10 : 1 : 1) of cytokinin extracts prepared from healthy (open circles) and infected (closed circles) seedlings in resistant host–parasite combination. The extracts were obtained from 70 g fresh weight of leaves/1000 ml medium

solvent consisted of *t*-butanol–ammonium hydroxide–H_2O (3 : 1 : 1). The extracts of healthy and infected seedlings contained a mixture of active materials. The data obtained by soybean bioassay (Fig. 3) reveal two peaks of cell division activity. The cell division factors in the extract from healty and infected plants at R_f 0.6 – 0.8 are chromatographically similar to Z, ZR and 2iP, IPA. The migration of active materials found at lower R_f regions do not correspond to the migration of any of the standards used. It is also seen that the cytokinin activity at R_f regions corresponding to Z and ZR is much higher in the extract of infected leaves than that in the healthy ones. In addition, the level of free base and nucleoside forms of 2iP in extract of infected plants is higher than that in the extract of healthy plants.

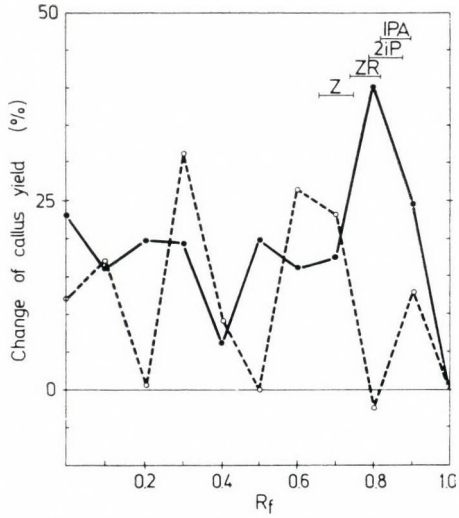

Fig. 3. The conditions as at Fig. 2 but chromatograms were developed in *t*-butanol–ammonia–water (3 : 1 : 1)

Discussion

Our results provide further evidence to the increased cytokinin level in a susceptible plant infected with an obligate parasite (cf. SEQUIERA, 1973). In addition, higher cell division activity has been found in a hypersensitive (resistant) combination than in the control healthy plants. However, this increase was lower than that in the susceptible combination. A similar result was found by VIZÁROVÁ (1974) in barley infected with powdery mildew. It is noteworthy that one can regularly observe the appearance of green islands not only in susceptible wheat-rust combinations but also in resistant ones. It is suggested that the high level of cytokinin in infected leaves is causally related to the appearance of the green islands. The origin of the increased cytokinin activity in both combinations remains to be determined.

It is interesting from this point of view, that a stimulated synthesis of sRNA was found in bean leaves infected with *Uromyces phaseoli* (HEITEFUSS and BAUER, 1969) and in rust infected oats (TANI *et al.*, 1971a). This is true for both susceptible and resistant combinations. The total nucleotide content of NaCl extractable RNA was also increased in rusted wheat (JOHNSON *et al.*, 1967). The increased RNA synthesis is accompanied by an increase in the RNase activity in rust infected wheat (ROHRINGER and HEITEFUSS, 1961; FRIČ and FUSCH, 1970; CHAKRAVORTY *et al.*, 1974).

One can point out that the picture of total ribonuclease activities in healthy and infected susceptible and resistant plants corresponds to the picture of cytokinin levels. This is in accord with the hypothesis that a close relationship exists between the production of cytokinins and the breakdown of nucleic acids (cf. HALL, 1973).

As regards disease resistance and cytokinin level in wheat, very few data were available in the literature (DALY, 1976). Efforts to prove causal relationship between IAA metabolism or ethylene production and resistance turned to be ineffective.

Exogenously added cytokinins increased rust resistance in detached leaf cultures (PERSON *et al.*, 1958; TANI *et al.*, 1971b; MAYAMA *et al.*, 1975). However, benzimidazole applied to intact plants did not effect resistance to stem rust though prevented the heat-induced susceptibility (BARNA, 1975). The loss of cytokinins in leaves leads to the breakdown of resistance to rust and this can be prevented by exogenously applied cytokinin treatment. Considering the facts that cytokinins influence many metabolic processes in the plant, and different concentrations of cytokinins can give different physiological reactions, we suppose that the question of the role of cytokinins in resistance is a complex problem and needs further detailed investigations.

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Effect of Nitrogen Supply and Peroxidase Enzyme Activity on Susceptibility of Wheat to Stem Rust

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Different levels of nitrogen (21, 630 and 1050 ppm) compared to the control (284 ppm) in Hoagland's solution did not change the infection type in the two terminal leaves of wheat (cv. Little Club) inoculated with *Puccinia graminis* f. sp. *tritici*, race 11 (infection type 3++ or 4).However, the number of infection sites was strongly influenced in the flag leaf and in the next leaf below the flag leaf by different nitrogen levels. The suboptimal nitrogen supply (21 ppm) significantly decreased the number of pustules; the two terminal leaves were more rust-resistant than the control leaves. These relatively resistant leaves had significantly lower peroxidase activity in the cell-free extract than did control leaves with the optimal nitrogen supply. Plants supplied with high rate of nitrogen exhibited about the same peroxidase activity as the control, in spite of the fact that their suspectibility (pustule number) was increased. The data suggest, that total peroxidase activity is not the cause of resistance at the adult plant stage, expressed either as infection type or as the number of pustules per unique leaf area.

It has been known for a long time, that high rates of nitrogen fertilizers may increase host susceptibility to various fungus diseases (CHESTER, 1946; DARLEY and HART, 1944; KIRÁLY, 1964; HUBER and WATSON, 1974). According to earlier investigators (GASSNER, 1915; STAKMAN and AAMODT, 1924; GASSNER and HASSEBRAUK, 1933; GASSNER and FRANKE, 1934; DARLEY and HART, 1944) the infection type to rust changes after high nitrogen doses in cereals. On the contrary, DALY (1949) found that mineral nutrition had little effect on reaction of susceptible and resistant cultivars. Only the mesothetic cultivar changed the reaction type. Presumebly in this latter case other resistance mechanisms are involved. The increased susceptibility expresses itself only in the increased number of rust pustules, thus, the infection type remains unaltered. This observation was strengthened by KIRÁLY (1964).

Several researchers have reported on increase in rust pustules with increased rates of nitrogen, however, no quantitative data as to numbers of pustules were reported, nor was there any attempt to find statistical correlation between pustule numbers and nitrogen levels.

Since peroxidase enzyme activity has been considered as a possible factor in determining horizontal (field) resistance, which accounts for the number of pustules, we examined the peroxidase activity of wheat leaves supplied with different rates of nitrogen.

Material and Methods

Seeds of wheat cultivar Little Club *(Triticum compactum)* were sown in clay pots filled with white silica sand, which had been watered with various solutions according to HOAGLAND and SNYDER (1933), containing various amounts of nitrogen. Following seedling emergence, all pots received 40 ml of their specific solutions twice a week. The sand was kept moist but not saturated in order to allow aeration of the root system. There were 5 pots of 10 plants each treatment. Three were inoculated with *Puccinia graminis*, race 11 at boot stage by brushing the two terminal leaves (flag leaf plus the subsequent leaf below the flag leaf) with a uredospore suspension. The other 2 pots were kept healthy for measurement of peroxidase activity. Since the form of nitrogen available to the host greatly effects resistance (HUBER and WATSON, 1974), we applied N both in nitrate and ammonium forms, but the majority of N was in the nitrate form.

Chemical composition of the solutions containing different levels of nitrogen is given in Table 1. All solutions were brought to the same osmotic value by adding NaCl. The minor elements were added by supplying 1 ml per liter of a solution containing 5.0 g ferric tartarate, 0.6 g H_3BO_3, 0.4 g $MnCl_2 \cdot 4H_2O$, 0.05 g $ZnSO_4$, 0.5 g $CuSO_4 \cdot 5H_2O$, and 0.02 g $H_2MoO_4 \cdot 4H_2O$. All solutions were adjusted to pH 6.0.

Pustule numbers were determined 14 days after inoculations and were expressed per cm^2 of leaf area.

Peroxidase enzyme assay. Five-g samples of leaf tissue from the different treatment were homogenized with 15 ml 0.15 *M* phosphate buffer (pH 7.0) and quartz sand in pre-cooled mortars at 4°C. The sap was expressed through four layers of cheesecloth and then centrifuged at 5500 *g* for 30 min at 0°C in a Janetzki

Table 1

Chemical composition of nutrient solution to obtain different levels of nitrogen (After CHEO *et al.*, 1952)

Salts	Molarity of solution	Amount (ml) of stock solution per liter of final solution to get concentration of N			
		21 ppm	630 ppm	1050 ppm	284 ppm+ (control)
$Ca(NO_3)_2.4H_2O$	2 5		2.0	2 0	3.02
KNO_3	2 0	0.75	2.5	2 5	2 52
$NaNO_3$	6.0		5.0		
NH_4NO_3	6 0			5.0	
KCl	3.0	1.16			
KH_2PO_4	1.0	1.00	1.0	1.0	1.30
$MgSO_4.7H_2O$	2.0	1.00	1.0	1.0	0.97
$CaCl_2.2H_2O$	4.0	1.25			
NaCl	5.0	8.00	2.0	2.0	

* Standard Hoagland's solution

K 23 centrifuge. The supernatant was then ultracentrifuged (50 000 *g* for 1 h) in a MOM G 120 analytical ultracentrifuge. The method for peroxidase assays was described earlier by BARNA *et al.* (1974).

Results and Discussion

The different levels of nitrogen contained by the various solutions supplied to the sand cultures of wheat plants exerted very conspicuous effects on the vegetative growth, which was first observed 2 weeks after sowing. The modified Hoagland's solution (see the chapter on Materials and Methods), which served as control, contained 284 ppm of nitrogen. At the 21 ppm level wheat plants were so deficient in nitrogen that they grew poorly, remained short in length and become yellow in color. Leaves were narrow and short and the lower leaves approached the senescent stage relatively early. Wheat plants treated with a 630 ppm concentration of nitrogen grew most rapidly. Their leaves were long, wide and had a dark green color. At the nitrogen level of 1050 ppm plants were dark green but noticeably stunted in comparison to the more optimal 630 ppm level of nitrogen (Fig. 1).

Fig. 1. The effect of different levels of nitrogen in sand culture on the vegetative growth of Little Club wheat. A: Hoagland's solution containing 21 ppm N; B: 630 ppm N; C: 1050 ppm N; D: Standard Hoagland's solution containing 284 ppm N

Susceptibility to stem rust. As it was expected nitrogen levels higher or lower than the control did not change the infection type of the two terminal leaves in this host-pathogen combination (infection type 3^{++} or 4). However, the number of rust pustules was strongly influenced by different nitrogen levels in the nutrient solution. Pustule numbers were calculated per cm^2 leaf surface and were expressed as percents of the control value. As is seen in Fig. 2 the suboptimal nitrogen supply significantly decreased the number of postules as compared to

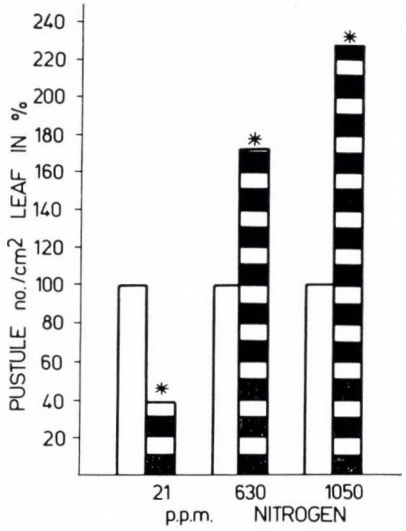

Fig. 2. Number of pustules of *Puccinia graminis* f. sp. *tritici* per 1 cm^2 leaf area of Little Club grown in sand culture and treated with modified Hoagland's solutions. The standard Hogland's solution served as control containing 284 ppm of nitrogen. White bars: control; striped bars: Hoagland's solutions containing different amount of nitrogen. The data represent 6 independent experiments. Pustule numbers were calculated as an average of 30 leaves in each experiment. Average of pustule number of the control flag leaves was 11.16/cm^2

the control (36.9% as compared to the 100% of control). In other words, the two terminal leaves of plants under nitrogen stress were more rust resistant than the leaves on control plants which received adequate nitrogen in the Hoagland's solution. Plants grown at 630 ppm nitrogen in the nutrient solution were significantly increased in susceptibility (174.5%). The increase in susceptibility was even higher at the highest nitrogen level (1050 ppm): 226.6% of the control. All of the differences were highly significant ($P = 0.01$).

Peroxidase activity in cell-free extracts of wheat leaves. Peroxidase activities of plants received different treatments neither parallelled degrees of resistance and susceptibility nor vegetative growths. As is seen in Fig. 3 the lowest activity of peroxidase was experienced in the cell-free extract of plants which received the lowest level of nitrogen in the nutrient solution. The value was 40.3% as compared to the control (100%). This difference was highly significant. However, plants

supplied with high doses of nitrogen exhibited about the same peroxidase activity as the control. In fact, the values in these cases were lower than the control value but differences were not significant.

The most important conclusion from these experiments was that wheat plants under nitrogen stress, although showing increased resistance (decreased susceptibility), had significantly lower peroxidase activity. In other words, resistance expressed on infection density did not correlate with increased peroxidase

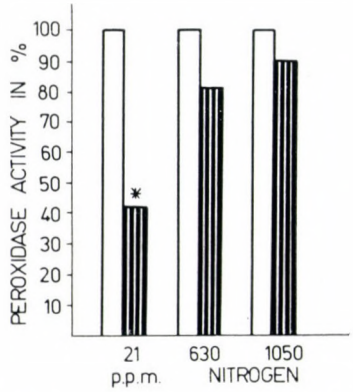

Fig. 3. The effect of different levels of nitrogen on the activity of peroxidase enzyme in leaves of Little Club wheat grown in sand culture and treated with modified Hoagland's solutions. White bars: control; striped bars: Hoagland's solutions containing different amount of nitrogen. The data are the average of 5 replications

activity. These experiments were convincing as to the secondary nature of activity of plant peroxidase in host–parasite relationships. In other words, our experiment contributed further data to the non-involvement of peroxidase activity in resistance of wheat to stem rust, expressed not only on infection type (DALY *et al.*, 1970; BARNA *et al.*, 1974) but also on the basis of reduced number of infection sites at adult plants.

Since the different levels of N may affect the protein metabolism of wheat leaves, we investigated the protein composition by polyacrylamide gel electrophoresis. However, although the total amount of protein increased with the increasing rate of nitrogen, we could not relate any qualitative changes in protein bands to the resistance against stem rust (cf. MASHAAL, 1976).

As early as 1934 GASSNER and FRANKE suggested that improved nitrogen nutrition increases susceptibility of cereals by increasing the formation of several specific proteins. The validity of this hypothesis was never supported experimentally, and the polyacrylamide gel electrophoresis used in our experiments was not suitable to demonstrate any changes in specific proteins after low or high nitrogen treatments.

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Changes in the Composition of Free and Protein Amino Acids in Groundnut Leaves Induced by Infection with *Puccinia arachidis* Speg.

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The changes in free and protein amino acids in rust-infected groundnut leaves during disease development, have been investigated by paper chromatography. The alterations in the free amino acid pool in infected tissues are given. Glutamic acid and threonine were eliminated gradually. The protein amino acids showed 1—3 fold increase in infected tissues during various stages of disease development.

Rust disease caused by *Puccinia arachidis* Speg. is one of the major diseases of groundnut in many countries of the world (JACKSON and BELL, 1969; PEREGRINE 1971; FEAKIN, 1973). In India it has been reported from Punjab, Tamil Nadu, Assam and Andhra Pradesh (BHAMA, 1972; RAMAKRISHNA and SUBBAIAH, 1973; CHOHAN, 1974; GOSWAMI, 1974). It became a potential menace for the sucessful growth of the crop and serious losses are met by the farmers every year. Despite this manifest importance certain areas of the problem have been neglected, particularly the physiology of the host-pathogen complex. Some of the biochemical changes in rust-infected groundnut leaves have already been reported by us (SUBRAHMANYAM *et al.*, 1976). The present work extends the investigation to the composition of free and protein amino acids during the cycle of the disease.

Materials and Methods

The leaves of groundnut (*Arachis hypogaea* L.) var. TMV2 plants were infected with *Puccinia arachidis* Speg. The method of growing plants and inoculating the leaves, as well as the type of infections which developed have already been described (SUBRAHMANYAM *et al.*, 1976). The disease progressed through five different stages — no visible symptoms (5 days after inoculation), small yellowish green circular lesions (8 days after inoculation), orange red pustules (10 days after inoculation), pustules dark brown (12 days after inoculation) and necrosis and yellowing of the leaves (15 days after inoculation). For extraction of the amino acids 1 *g* samples of uninfected and infected leaves were collected at these different stages of disease development, chopped into pieces, extracted with hot 80% ethanol and centrifuged. The supernatent was evaporated to dryness and used for free

amino acids. The residue was hydrolysed with 6*N* HCl in sealed tubes at 120°C for 24 hrs. The acid was removed by evaporation and used for protein amino acids. The residues left over after evaporation of the two fractions were dissolved in known quantities (1 ml) of 80% ethanol and stored in tightly stoppered vials at 4°C until processed for chromatography.

The amino acids were separated by 2-dimensional ascending paper chromatography using *sec.* butanol : formic acid : water (75 : 13 : 12) and buffer saturated phenol as the first and second solvent systems, respectively (TURBA, 1954). The dried sheets were sprayed with 0.2% ninhydrin in *n*-butanol. The identity of the spots was established by co-chromatography and also by comparing the R_f values of the amino acids in a known mixture, with those in the extract. The individual spots were eluted and estimated quantitatively according to the method of GIRI *et al.* (1952). The quantity of individual amino acids was then determined by reference to their respective standard curves.

Results

There was a progressive change in the composition of free and protein amino acids throughout the course of rust development. The extent of such alterations is illustrated by the data in Tables 1 and 2. The nine free amino acids detected

Table 1

Effect of rust-infection on free amino acid content (μg/g fresh wt.) of healthy and infected groundnut leaves at various stages of disease development*

Amino acid	Stage 1		Stage 2		Stage 3		Stage 4		Stage 5	
	H	I	H	I	H	I	H	I	H	I
Aspartic acid	30	32	40	35	60	20	50	35	50	25
Glutamic acid	50	45	40	20	55	15	30	T	20	—
Serine	60	60	50	30	70	20	40	30	35	10
Threonine	25	28	30	25	30	T	20	—	10	—
Alanine	65	74	60	130	40	190	50	220	70	245
Tyrosine	30	36	35	60	40	50	65	90	60	100
γ-Amino butyric acid	40	38	30	35	35	40	30	55	30	50
Valine	T	T	T	10	10	30	20	60	35	65
Phenylalanine	T	T	T	20	10	25	25	60	20	75
Total (μg)	300	313	285	365	350	390	330	550	330	570
Total in % of control	100	104.33	100	128.07	100	111.43	100	166.66	100	172.73

H = Healthy I = Infected T = Present in traces only
— = Completely absent
* = Each figure is an average of two replicates

Table 2

Effect of rust-infection on protein-bound amino acid content (mg/g fresh wt.) of healthy and infected groundnut leaves at various stages of disease development*

Amino acid	Stage 1		Stage 2		Stage 3		Stage 4		Stage 5	
	H	I	H	I	H	I	H	I	H	I
Aspartic acid	0.25	0.20	0.30	1.10	0.35	1.00	0.28	0.95	0.30	0.86
Glutamic acid	0.90	0.85	0.80	1.30	0.96	1.20	0.85	1.65	0.78	1.80
Serine	0.75	0.70	0.67	1.30	0.70	1.10	0.72	1.25	0.69	1.40
Glycine	0.75	0.82	0.60	0.85	0.95	1.05	1.10	1.20	1.15	1.35
Histidine	0.50	0.55	0.75	0.80	0.68	1.20	0.72	1.10	0.70	1.32
Arginine	0.45	0.48	0.60	1.00	0.85	1.05	0.80	1.10	0.95	1.25
Threonine	0.70	0.75	0.90	1.05	1.10	1.30	1.25	1.42	1.19	1.40
Alanine	1.95	1.88	1.40	1.80	1.20	1.80	1.36	1.95	1.20	2.10
Tyrosine	0.30	0.35	0.28	0.30	0.19	0.45	0.14	0.20	0.15	0.50
Proline	0.10	0.12	0.15	0.10	0.18	0.30	0.20	0.20	0.19	0.23
Trytophan	0.50	0.56	0.85	1.40	1.05	1.60	1.10	1.20	1.10	1.20
Valine	0.55	0.60	0.60	0.80	0.75	0.93	0.85	0.95	0.92	1.20
Phenylalanine	0.65	0.70	0.80	1.20	0.60	1.38	0.63	1.45	0.73	1.50
Leucine/*iso*Leucine	1.15	1.20	1.45	1.60	1.52	1.90	1.60	2.10	1.55	2.45
Total (mg)	9.50	9.76	10.15	14.60	11.08	16.26	11.60	16.72	11.60	18.56
Total in % of control	100	102.73	100	143.84	100	146.75	100	144.13	100	160.00

H = Healthy I = Infected
* Each figure is an average of two replicates

were aspartic acid, glutamic acid, serine, threonine, alanine, tyrosine, γ-amino butyric acid, valine and phenylalanine. In healthy plants the contents of aspartic acid, tyrosine and valine increased, of glutamic acid and serine decreased while the others fluctuated. In infected plants alanine, tyrosine, γ-amino butyric acid, valine and phenylalanine tended to show increase in their quantity as disease progressed, while the remaining were much affected and showed decreases in their quantities. Glutamic acid and threonine were eliminated gradually (Table 1).

The protein hydrolysates of both healthy and infected plants contained 14 ninhydrin positive compounds (Table 2). The amino acids found in the protein hydrolysate and not detected in the free amino acid pool were glycine, histidine, arginine, proline, tryptophan and leucine/*iso* leucine. γ-Amino butyric acid was absent from the protein amino acid pool. The remaining acids were common to both free and bound amino acid pools. Protein amino acids occurred in much greater quantities than did free amino acids in the same tissues. In healthy plants glycine, histidine, arginine, threonine, proline, tryptophan, valine and leucines showed gradual increase in their quantities while the others fluctuated. Without exception all the protein amino acids increased as a result of rust infection and this increase ranged from 1—3 fold with different amino acids (Table 2).

These changes that are observed in the amino acid spectrum allow us to conclude that the amino acid metabolism of groundnut leaves significantly changes as a result of pathogenic invasion by rust.

Discussion

Physiology of rust-infected plants have been published by several workers. All these studies were made mainly with regards to starch and carbohydrates and the nitrogen compounds have received sparse attention (BUSHNELL, 1970). However, changes in amino acid compounds in the various phases of disease induced by obligate parasites have been studied by some authors (ROHRINGER, 1957; SIEBERT, 1961; SHAW and COLOTELO, 1961; RUDOLPH, 1963; RAGGI and SEMPIO, 1964; SEMPIO and RAGGI, 1966; RAGGI, 1974). It is possible to reconcile our results with them, who also observed an increase in the total free amino acid content in the infected tissues as compared to the healthy controls (Table 1). The striking diminution of glutamic acid and threonine was also noted in the results of SHAW and COLOTELO (1961) and RUDOLPH (1963). The decrease in glutamic acid content may be related to the increased γ-amino butyric acid because it is generally derived from glutamic acid by decarboxylation. The increase in phenylalanine (also tyrosine) may be correlated with the acceleration of phenolic acid metabolism of rust-infected tissues as suggested by ROHRINGER *et al.* (1967) and FUCHS *et al.* (1967). As it belongs to the shikimate pathway (NEISH, 1964) its increase was attributed to the activation of this cycle. A study of the phenolic acid metabolism of rust-infected plants would be useful in this respect.

The increase of certain amino acids in the infected tissues may be due to the *de novo* synthesis by the host (ROHRINGER, 1957) or proteolysis of certain host tissue proteins (KIRÁLY and FARKAS, 1959; RUBIN and ARTSIKHOVSKAYA, 1963) or synthesis by the pathogen itself (VAN ANDEL, 1966; ROHRINGER, 1957). The increase may also be attributed to the activation of enzymes involved in amino acid and amide synthesis (GOODMAN *et al.*, 1967). Further, by using both chemical and radioactive tracer techniques several workers have demonstrated an accumulation of metabolites at the infection sites of obligate parasites. The diminution of some of the amino acids may be attributed to their utilization by the parasite or to their diversion to some other mechanism in the altered metabolism.

Variations in the composition of protein amino acids substantially agree with those of SHAW and COLOTELO (1961) who also registered a notable increase in protein amino acids in the rust infected plants with respect to the healthy ones. This increase may indicate an enhanced synthesis of protein in the infected tissues. Generally, in fungus-infected plants the total nitrogen and protein content of the host-pathogen complex increase (GOODMAN *et al.*, 1967). SHAW and COLOTELO (1961) observed an increase of total nitrogen, protein nitrogen and soluble nitrogen as the rust developed. The rate of increase of different protein amino acids is not uniform and this may indicate that some qualitative (along with quantitative)

changes of proteins might be occurring in the host tissues with the progress of disease. SHAW and COLOTELO (1961) demonstrated the formation of a new protein as a reaction of the host to infection. Since there is increase of both protein and free (at least some) amino acids, it indicates that the amino acids must be translocated from other parts of the host to the infected tissues (SHAW and COLOTELO, 1961; RUBIN and ARTSIKHOVSKAYA, 1963) or amino acid synthesis must be increased as well as protein synthesis (SHAW and COLOTELO, 1961).

It would be of great importance to study the alterations in proteins (quantitative and qualitative, if any) and enzymes involved in the amino acid metabolism, of rust-infected groundnut leaves. Such studies may add further to the better understanding of the observed changes in the protein and free amino acid pools of the infected tissues.

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Scanning Electron Microscopy of Lettuce Leaves Inoculated by *Bremia lactucae* Regel

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The preparation of lettuce leaves for scanning electron microscopy (SEM) after inoculation with *Bremia lactucae* is described.

Conidium germination, formation of appressoria, and the evidence of host penetration either directly or through stomata has often been found in compatible and incompatible combinations, respectively. In some cases, however, irregular germ tubes and double appressoria have formed indicating the lack of affinity between host and pathogen in that case.

The advantages of examination by SEM are also discussed.

The scanning electron microscope has recently been used in the field of mycology and phytopathology, but it seemed to be soon a promising instrument for observing fungal pathogens on leaf surfaces (BARNES and NEVE, 1968). This new technique offers several advantages over the light microscope, namely a very good resolution, a wide range of magnifications, as well as a large depth of focus, giving a three dimensional effect of the pictures (BARNES and NEVE, 1968; IDLE, 1969). One of the difficulties of the application for biological material is the long preparation of the specimens during which fungal elements and leaf surfaces can easily be damaged. This fact may explain the low number of publications, especially those dealing with the fungi of Peronosporaceae (LOCCI, 1969; ROYLE and THOMAS, 1971; SHIRAISHI *et al.*, 1975).

In this paper observations using the SEM technique are reported and the infection process of *B. lactucae* is illustrated.

Materials and Methods

Inoculation

Discs, 10 mm in diameter, were cut from well developed leaves of lettuce plants, cv. "Caravan" and "Solito" grown in a glasshouse. They were placed

on to wet filter paper in Petri dishes and inoculated by spraying with conidium suspensions of *Bremia lactucae* races NL−2, NL−3 and NL−5, respectively. The inoculum concentration was adjusted to $3-4 \times 10^5$ conidia/ml.

Preparation of the specimens

Leaf discs were taken 10 and 24 hours after inoculation and fixed for 16−22 hours in 6.25% glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.0) at 4°C. After rinsing three times in the same buffer at 4°C over 1 hour, the discs were slowly dehydrated in ethanol : water series graded at 10% intervals. Dehydration was followed by impregnation with amyl acetate, using a series of four amyl acetate : absolute ethanol graded solutions. In order to avoid distortion of the material small bags made from synthetic mesh were used for holding the specimens. The discs were then dried by the "critical point" method where amyl acetate was replaced by liquid CO_2 which then evaporated from the specimens when heated to slightly above its critical point (32°C and 83 atm). Each disc was glued to a metal stub and coated with gold by evaporation under vacuum, using the sputter technique, to make the surface electrically conductive.

The specimens were examined under the scanning electron microscope JSM−U3) with a beam voltage of 25 kV.

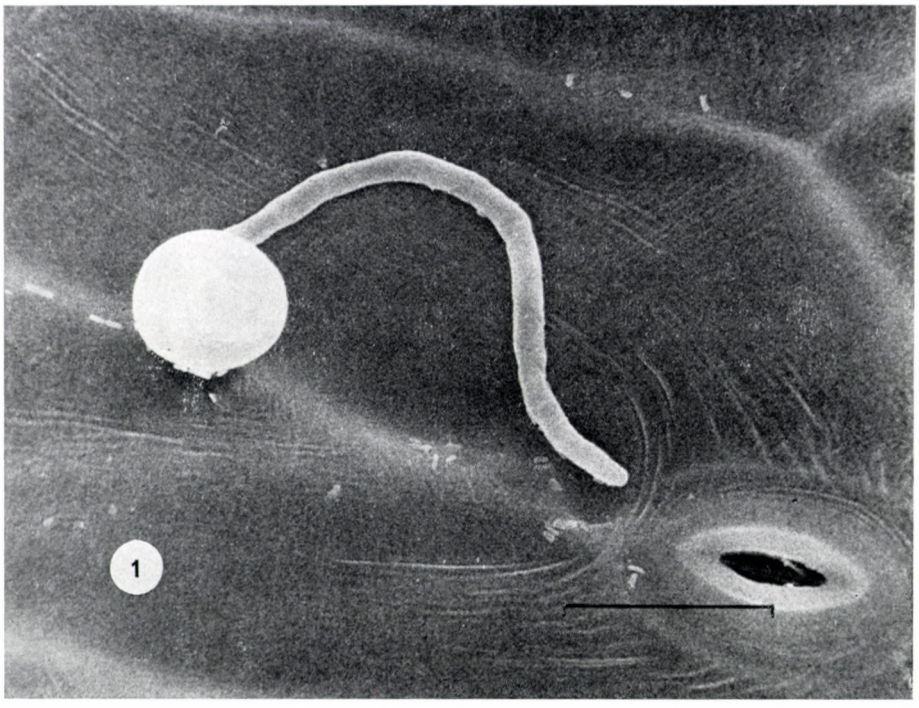
Results

Various types of conidium germination are illustrated in Plate I, II and III. Firstly a thin germ tube develops from the conidium (Plate I, Fig. 1), and its end expands forming an appressorium (Plate I, Fig. 2). Germination of *B. lactucae* conidia usually takes place in such a way. Nevertheless, the length of the germ tube seems to be in correlation with the affinity between host and pathogen (Plate II, Figs 1 and 2). Thus very long germ tubes with branching (Plate III, Fig. 1), moreover double appressorium showing further growth at its end (Plate III, Fig. 2) are mainly characteristic of the specimens from incompatible combinations (NL−2 on Solito).

Direct penetration of the leaves is very typical of *B. lactucae* (Plate IV, Fig. 1), while stomata, even if they are open, are usually avoided, or overgrown by the germ tubes (Plate IV, Fig. 2). Plate V clearly shows that despite of the vicinity of stomata the fungus prefers direct penetration through the cuticule.

Based on a large number of examinations under the SEM it can be ascertained that the penetration site is localized at the joining point of two or more cell walls (Plate II, Fig. 1; Plate V, Figs 1 and 2; Plate VI, Figs 1 and 2). In Plate VI, Fig. 2 a penetration, taking place in the ditch of such a joining point, is illustrated.

In a few cases germ tubes enter the host plant by growing into the stomatal openings (Plate VII, Figs 1 and 2). The micrographs also demonstrate that in case of penetration through a stoma no appressoria are formed.



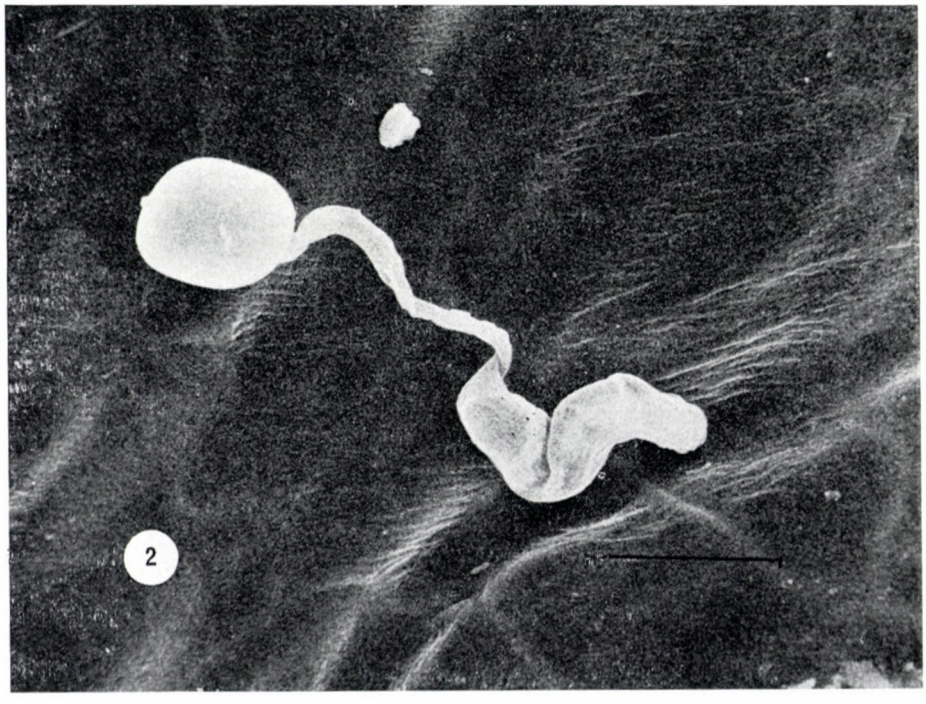


Plate I, Figures 1 and 2. Germinating conidia of *B. lactucae* on lettuce leaves with and without appressorium

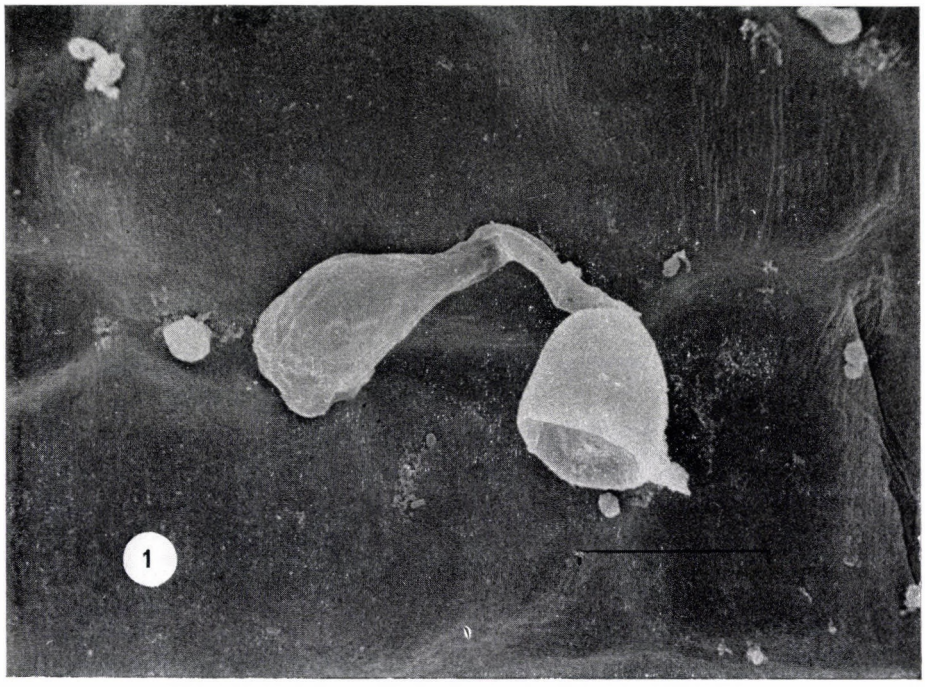


Figure 1. Short germ tube and appressorium (compatibility)

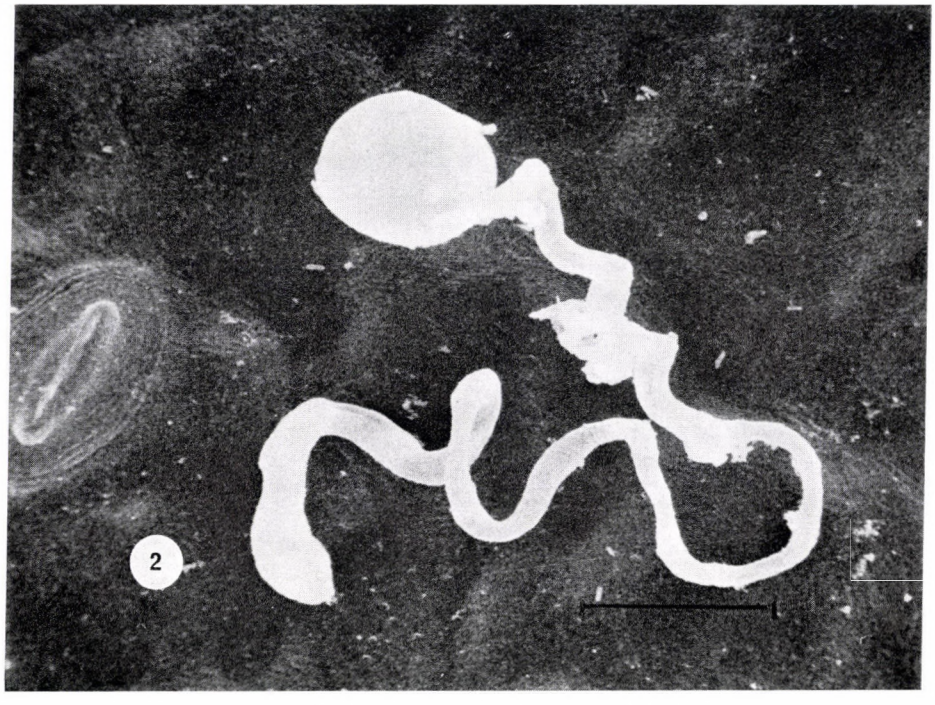


Figure 2. Long germ tube (incompatibility)

Plate II, Germinating conidia of *B. lactucae* on lettuce leaves

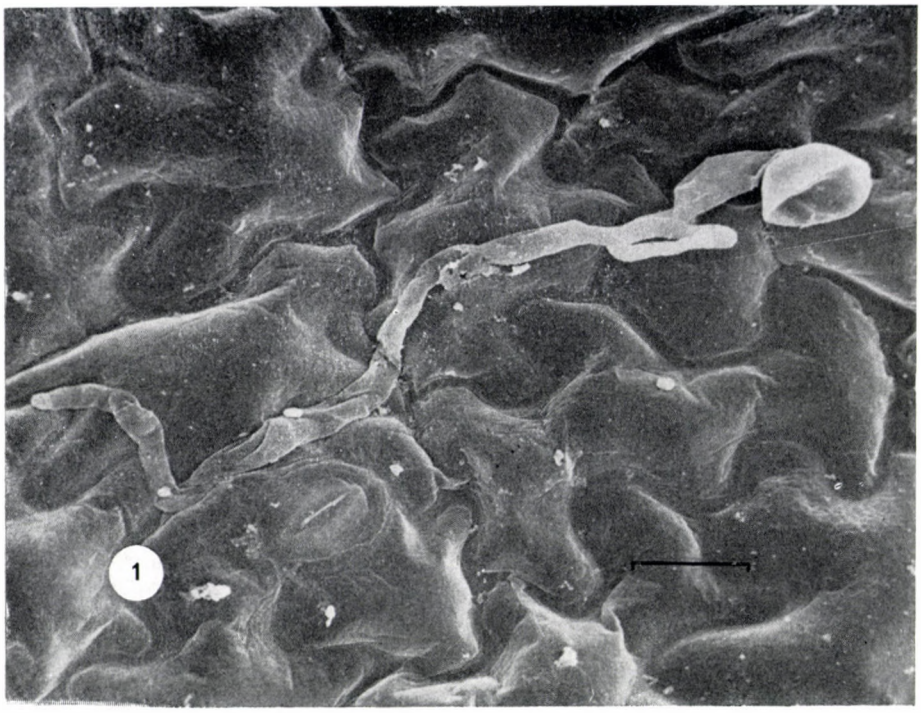


Figure 1. Unu ually long germ tube with branching

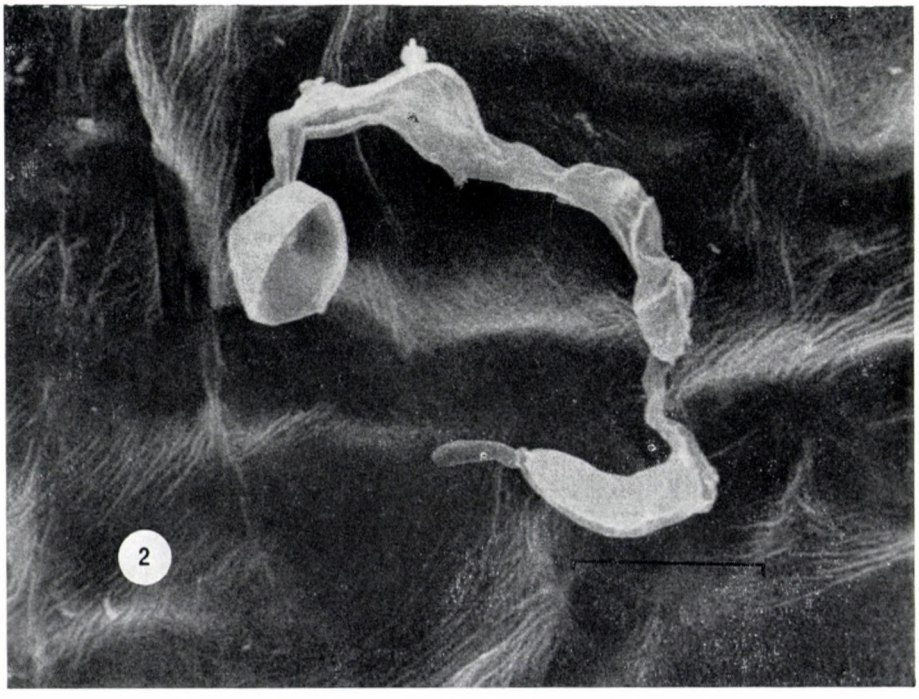


Figure 2. Double appressorium and secondary germ tube

Plate III, Types of irregular germination

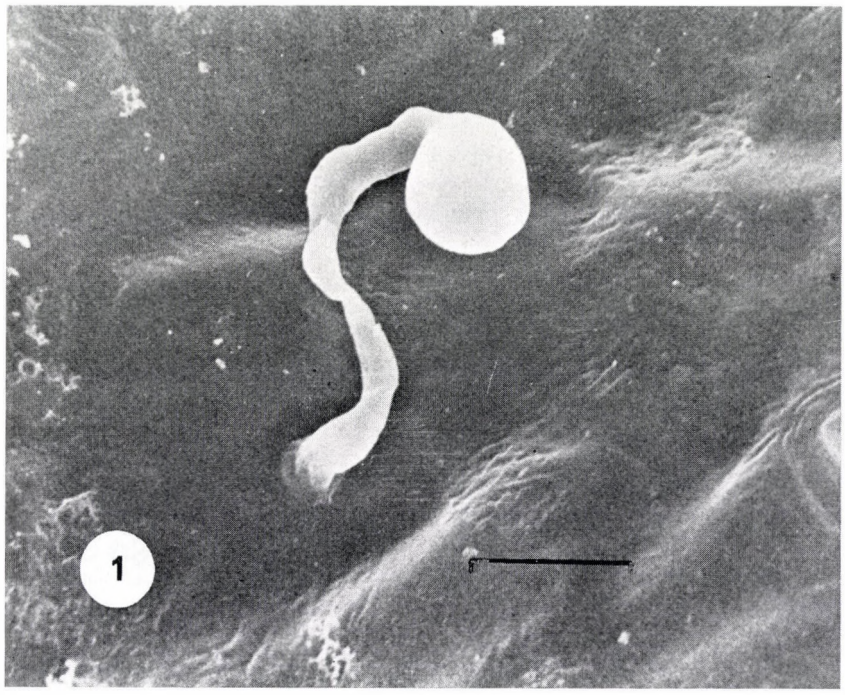


Plate IV, Figure 1. Direct penetration

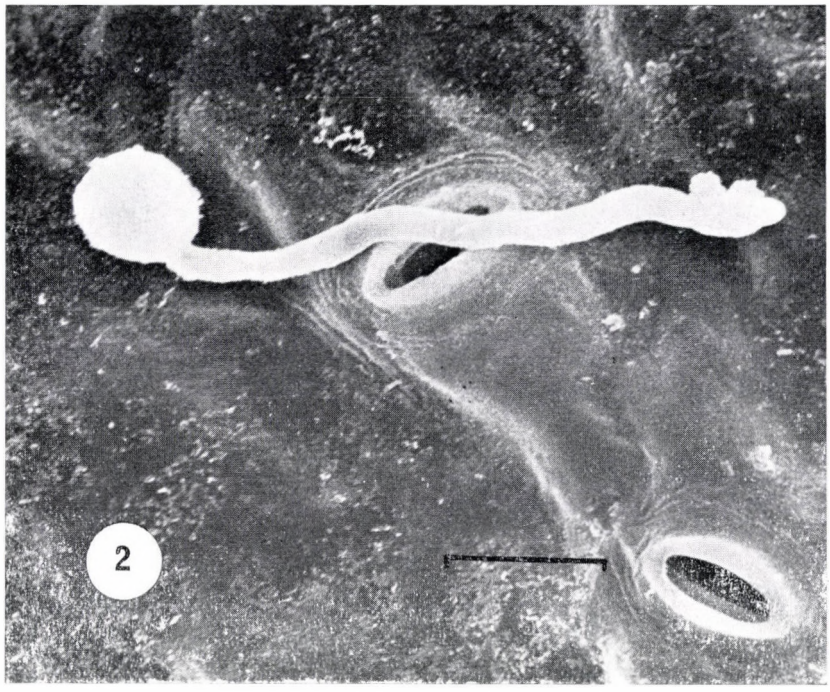


Figure 2. Stoma overgrown by a germinating conidium

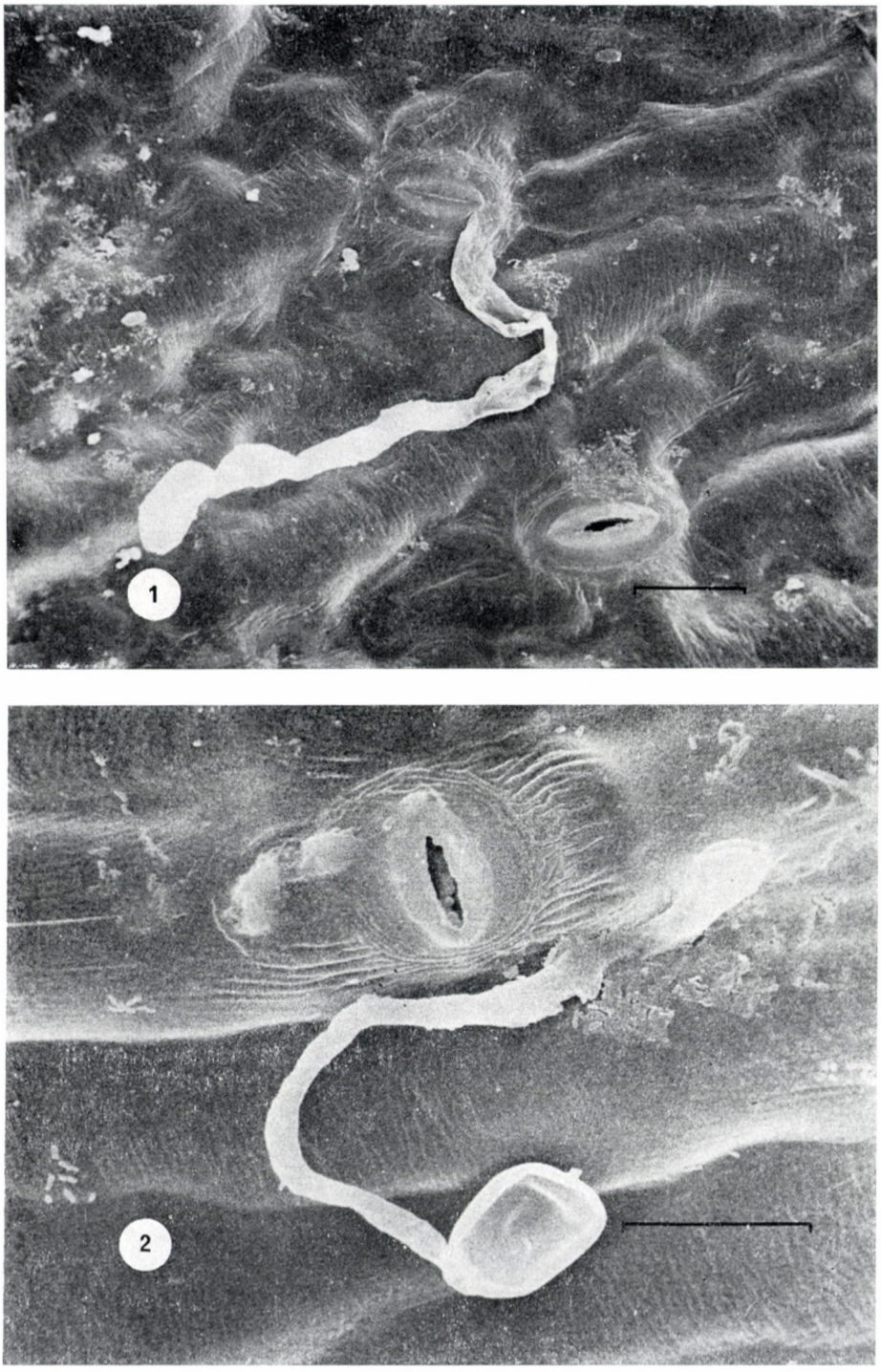


Plate V, Figures 1 and 2. Direct penetration in the vicinity of stomata

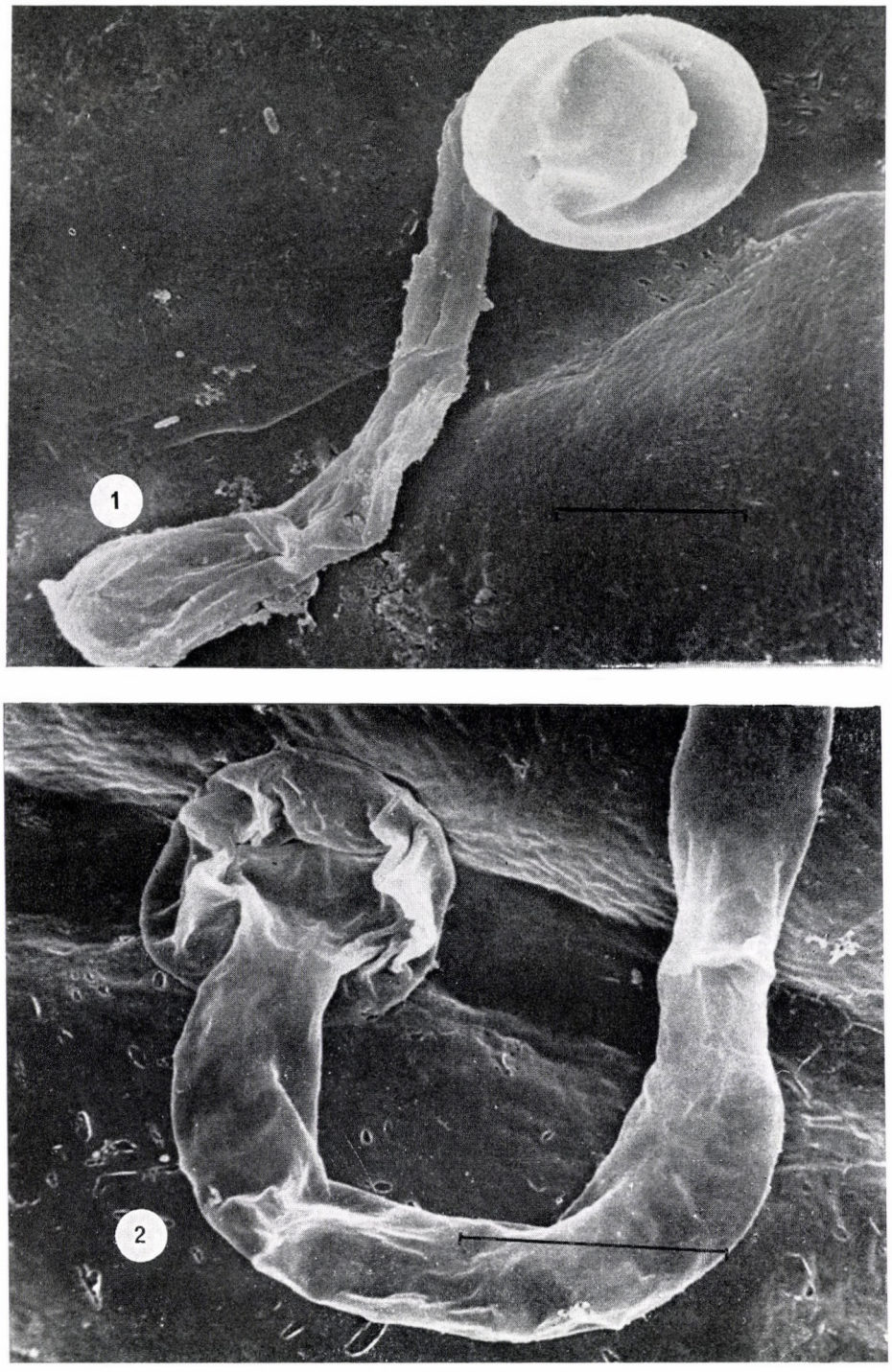
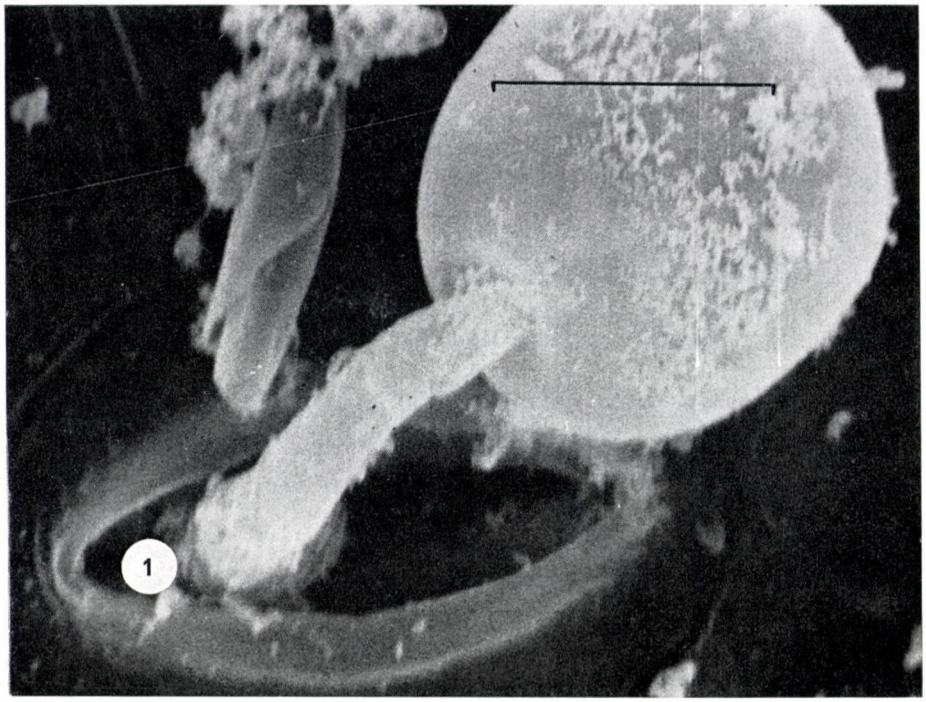


Plate VI, Figures 1 and 2. View of penetration sites at the joining of cell walls



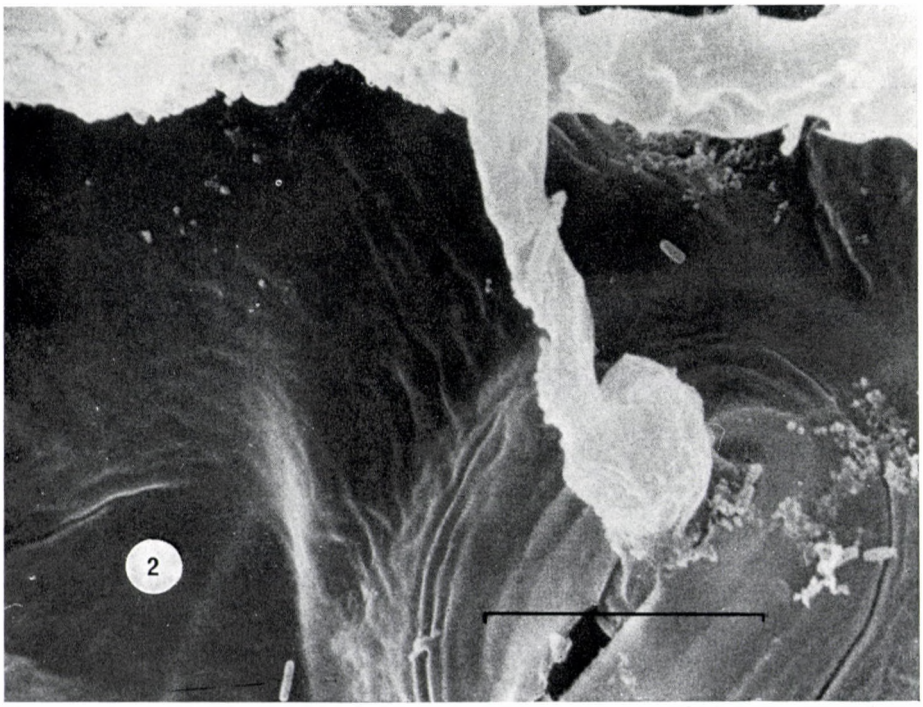


Plate VII, Figures 1 and 2. Penetration through stomata
Scale: bar = 20 μm, except for Plates VI and VII (bar = 10 μm)

Discussion

According to LOCCI (1969) one of the advantages of the SEM technique is the short time needed for preparation of the specimens. That is true when fixation and dehydration are omitted, as it was done by LOCCI (1969), whereas the method used by the authors needs more time. The mode of fixation, dehydration, embedding and drying was similar to that described by ROYLE and THOMAS (1971) and proved to be the best in the present work.

It is interesting to note that each specimen, belonging either to compatible or to incompatible combinations, have shown fungal penetration. The incidence of irregular germ tubes was the only sign of incompatibility.

In agreement with BARNES and NEVE (1968) and others it was found that the SEM technique is suitable for studying fungal pathogens on the plant surface. Moreover this method can provide new information about morphological and physiological aspects of host–pathogen relations.

Acknowledgements

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Comparison of Esterase Patterns of *Fusarium culmorum* and *Fusarium graminearum*

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Acetylesterase (E.C. 3.1.1.6.) patterns of 35 isolates of *Fusarium culmorum (W. G. Smith) Sacc.* and *F. graminearum Schwabe* originated from different geographic locations and different sources were compared. Fifteen esterase bands with different R_f value were identified. These 15 esterase bands occurred both in *F. culmorum* and *F. graminearum*. None of them proved to be species specific. Degree of homology and heterology between two species were established with statistical methods (*binary contingency tables*, and X^2-*statistics*) on the basis of the frequency distribution of esterase bands. The *average of D% values* ($\overline{D\%}$) between the two species was *21.5*, while the *average similarity* ($\overline{S\%}$) was 79.5. The X^2-*test* presented in case of $df = 14$ at $P = 0.1$ probability level a strong significant difference between the two species. The degree of this difference was expressed to the value of *21.5%*. The *F. culmorum* and *F. graminearum* could not be separated from one another in practice by the esterase pattern method.

The classification of fungi is based primarily on morphological features (AINSWORTH, 1971). Other criteria derived from a wide range of techniques involving some type of chemical analysis have been tried (GARBER and RIPPON, 1968; HALL, 1969; TYRELL, 1969). One of these techniques is gel electrophoresis of proteins and enzymes extracted from the mycelium and spores or culture filtrates of fungi. Cellular extracts or culture filtrates have been used for electrophoresis and the comparisons here involved either patterns of sites of stained protein *(protein profiles)* or patterns of sites of enzyme activity *(zymograms)*. The zymogram (HUNTER and MARKERT, 1957) in starch or acrylamide gels separates enzymes according to their electrical charges and molecular dimensions and indicates their activity by specific staining. Procedures for obtaining zymograms for a number of enzyme systems, recently was reviewed by BREWER and SING (1970), and SHAW and PRASAD (1970).

Enzyme patterns (zymograms) obtained from various fungal components and culture filtrates of different species have been used as a taxonomic criterion, using such enzyme systems as esterase, leucine aminopeptidases, phosphatases and several dehydrogenases: *Myxomycetes* (FRANKE, BALEK and VALENTIN, 1968; FRANKE and BERRY, 1972; BERRY and FRANKE, 1973), *Oomycetes* (WANG and

LÉJOHN, 1974a, b, c), *Phytophthora sp.* (CLARE *et al.*, 1968), *Mucor sp.* (STOUT and SHAW, 1974), *Thamnidium sp.* (STOUT and SHAW, 1973), *Candida sp.* (BERCHEV and IZMIROV, 1967), *Neurospora sp.* (REDDY and THRELKELD, 1971 and 1972; REDDY, 1973), *Gaeumannomyces sp.* (ABBOTT and HOLLAND, 1975), *Sclerotinia sp.* (WONG and WILLETTS, 1973 and 1975), *Colletotrichum sp.* (HENRY and GARBER, 1967), *Aspergillus sp.* (NEALSON and GARBER, 1967; GARBER, 1974), *Verticillium sp.* (SELVARAJ and MEYER, 1974), *Humicola sp.* (MOORHOUSE and DEBERTOLDI, 1975), *Ustilago sp.* (BRADFORD *et al.*, 1975).

Fusarium is one of the most heterogenous and taxonomically the most difficult fungal genera. The species of *Fusarium* are ubiquitous organisms encountered both as pathogens and saprophytes throughout the world. The present systems of *Fusarium* classification is based mostly on morphological characters (BILAI, 1955 and 1970; BOOTH, 1971; GERLACH, 1970; GORDON, 1960; JOFFE, 1974; MATUO, 1961; MESSIAEN and CASSINI, 1968; RAILLO, 1949; SNYDER and HANSEN, 1940, 1941, 1945; SNYDER and TOUSSOUN, 1965; WOLLENWEBER and REINKING, 1935). The *Fusarium* genus, however, is notoriously difficult to classify using the methods of classical mycology because the lack of morphological stability and uniformity of species within the group. Because identification of Fusaria may be difficult using orthodox techniques BOOTH (1966), suggested the use of biochemical methods. MEYER *et al.* (1964) examined the patterns of esterase and phosphatase in culture filtrates of 8 formae belonging to *F. oxysporum* and *F. xylaroides* and reported that differences appeared to be characteristic of the taxa. HALL (1967) had shown that catalase isozyme profiles distinguished cultural types within *F. solani* f. sp. *pisi*. GLYNN and REID (1969) in a study of 14 species of Fusaria with gel electrophoresis, reported that their result do not support the idea that the gel electrophoretic technique is of taxonomic value. MEYER and RENARD (1969) studied the esterase and protein patterns of various strains belonging to *F. oxysporum* f. sp. *melonis*, and *F. oxysporum* f. sp. *elaeidis*, and found that differences between the strains are as great as the differences between the formae. DRYSDALE and BRATT (1971) studied the esterase, peroxidase and acid phosphatase patterns from five strains of *F. graminearum*. They concluded that the polyacrylamide gel electrophoresis distinguished the members of this particular group of isolates of *F. graminearum*. REDDY and STAHMANN (1972) attempted to extend these studies to the patterns of thirteen enzymes with a special emphasis on dehydrogenases and to develop some criteria for the identification of ten fungi belonging to 5 species of *Fusarium*. They reported that among 13 different enzymes malate dehydrogenase, peroxidase, acid phosphatase, glucokinase and acetylesterase had a species and forma characteristic patterns. They concluded from their results these patterns either alone or in combination could be used as useful taxonomic criteria.

Regarding to papers published till now we make the following reflections:

1. Few data were reported on numerical analysis of electrophoretic protein and enzyme patterns of a fungal genus: *Mucor* (STOUT and SHAW, 1974), *Candida* (SHECHTER *et al.*, 1972), *Taphrina* (SNIDER and KRAMER, 1974).

2. In most cases one isolate represents one species, and differences found between the strains were used taxonomic characterization of the species.

3. Few data were reported on the range of variability within a species due to the analysis of only a few isolates of a single species, the use of only a limited number of pattern variations within a taxon (GARBER and RIPPON, 1968; BERRY and FRANKE, 1973).

In the present investigations we compared *acetylesterase (E. C. 3.1.1.6.)* patterns of 35 isolates of *Fusarium culmorum (W. G. Smith) Sacc.* and *Fusarium graminearum Schwabe.* We determined the degree of difference between two species according to esterase patterns. We wanted to decide whether the esterase pattern method can be used to identify these species in practice.

Materials and Methods

Source of isolates. The *F. culmorum* and *F. graminearum* isolates used in these studies are listed with sources in Table 1 and Table 2. The strains isolated by us were determined according to BOOTH (1971).

Culturing of isolates. The isolates were grown in 100-ml Erlenmeyer flasks containing 30 ml standard medium composed of peptone (Difco), 10 g; yeast extract (Oxoid), 3 g; D-glucose (Reanal), 20 g in 1 liter of destilled water at pH 6.5. Cultures were incubated for 10 days at room temperature (22–24 C).

Preparation of cell-free extracts. The mycelia were harvested on a Büchner funnel, using four layers of cheese cloth, washed five times with tap water and one times with destilled water. The wet weight of the mycelia was 3–6 g depending on the isolates. This material was than frozen and stored at −15°C until used. No significant differences were observed between patterns of esterase extracted from freshly harvested fungal material and that stored in the freezer for up to 4 weeks. The frozen mycelia was first ground to a powder using a prechilled mortar and pestle in a cold place (5°C). After the mycelium powder was frozen again and it was second ground with acid-washed sand using a prechilled mortar and pestle in a cold place. The soluble proteins were then extracted by the addition to the mortar of cold modified *0.01 M tris-glycine buffer* (1g : 5 ml) and occasionally stirred. This buffer consisted of *0.01 M tris-glycine*, *0.25 M sucrose* and *0.0033 M* $CaCl_2$ and had a *pH of 8.3* (FRANKE and BERRY, 1972). The suspension was stirred with a magnetic stirrer at 4°C for 45 minutes. Then the resultant slurry was centrifuged at 5.000 rpm at 0°C for 45 minutes. The supernatant was saved and the cell debris discarded.

This preparation was used as the source of esterase for gel electrophoresis. This preparation was then frozen and stored at −15°C until used.

Disc gel electrophoresis. The gels used in electrophoresis were mixed according to the procedure of DAVIS (1964) except that *0.46 ml* of *TEMED (N,N,N¹,N¹-tetramethylethylene diamine)* from Eastman Kodak Co. were used in *Solution A; 25.6 ml of 1 M* H_3PO_4, *5.7 g* of *tris* were used in *Solution B (pH 6.9);* and *15 mg*

Table 1

2×2 contingency table for comparison the frequency distributions of individual esterase bands of isolates of *Fusarium culmorum* and *F. graminearum*

	R_{f_j}		
	S_1	S_0	Total frequency
Fusarium culmorum	a	b	$a+b$
Fusarium graminearum	c	d	$c+d$
Total frequency	$a+c$	$b+d$	N

$$x^2 = \frac{N\left([ad - bc] - \frac{N}{2}\right)^2}{(a + c)(a+b)(c+d)(b+d)}$$

$df = 1$

Table 2

Contingency table with X^2-test for the homogeneity of two frequency distributions in the case of 15 classes

Esterase band	F. culmorum a_j	F. graminearum b_j	a_j+b_j	$\frac{a_j}{a_j+b_j}$	$a_j\frac{a_j}{a_j+b_j}$
1. ↓ 15.					
	n_a	n_b	n_a+n_b	$\frac{n_a}{n_a+n_b}$	$Ea_j\frac{a_j}{a_j+b_j}$

$$X^2 = \frac{E\frac{a_j}{a_j+b_j} - \frac{n_a^2}{n_a+n_b}}{\frac{n_a}{n_a+n_b} - \left[\frac{n_a}{n_a+n_b}\right]^2}$$

$df = 14$

of $K_3Fe(CN)_6$ and *44* g of *acrylamide* (Sigma) to adjust the final separating gel to *11%* *acrylamide* were incorporated into *Solution C*. The 11% acrylamide concentration gave the highest resolution and greatest number of bands (FRANKE and BERRY, 1972). A sample gel was not used. The soluble protein content of samples determined by the microbiuret method (BAILEY, 1967). The volume of each sample necessary to give about *400 γ* of protein for electrophoresis was calculated. Electrophoresis was carried out on a custom built electrophoretic apparatus (Reanal, Hungary) at 4°C using a 0.01 M tris-glycine buffer, pH 8.3 (without sucrose and $CaCl_2$). The upper buffer chamber contained *2 ml* per liter

Table 3

Fusarium culmorum isolates and their sources

Isolate number	Origin	Source[a]
1	Corn root (Martonvásár, Hungary)	Á. Szécsi, 1971
2	Corn root (Martonvásár, Hungary)	Á. Szécsi, 1971
3	Corn stalk (Martonvásár, Hungary)	Á. Szécsi, 1971
4	Corn root (Bóly, Hungary)	Á. Szécsi, 1970
5	Corn stalk (Bóly, Hungary)	Á. Szécsi, 1968
6	Corn stalk (Bóly, Hungary)	Á. Szécsi, 1968
7	Corn root (Baja, Hungary)	Á. Szécsi, 1971
8	Corn stalk (Baja, Hungary)	Á. Szécsi, 1971
9	Corn root (Szeged, Hungary)	Á. Szécsi, 1972
10	Corn stalk (Szeged, Hungary)	Á. Szécsi, 1972
11	Wheat root (Mezőtárkony, Hungary)	Á. Szécsi, 1970
12	Wheat ovum (Keszthely, Hungary)	L. Hornok, 1972
13	Wheat ovum (Keszthely, Hungary)	L. Hornok, 1972
14	Wheat leaf (Kaba, Hungary)	L. Hornok, 1970
15	Hop root (Gödöllő, Hungary)	L. Hornok, 1970
16	Hop root (Gödöllő, Hungary)	L. Hornok, 1970
17	Hop root (Gödöllő, Hungary)	L. Hornok, 1970
18	Potato tuber (Budapest, Hungary)	L. Hornok, 1974
19	Potato tuber (Budapest, Hungary)	L. Hornok, 1974
20	Potato tuber (Budapest, Hungary)	L. Hornok, 1974
21	Lucern root (Kompolt, Hungary)	L. Hornok, 1972
22	Barley root (Ireland)	DSM-IMB 11359, Gerlach, 1968
23	Soil (England)	DSM-IMB 11425, Taylor, 1970
24	Corn stalk (Switzerland)	DSM-IMB 11672, Lazarowicz, 1969
25	Corn stalk (German Federal Rep.)	DSM-IMB 11722, Gerlach, 1971
26	Oat (German Federal Republic)	CBS 173.31, Wollenweber
27	Wheat (Netherlands)	CBS 251.52
28	Barley (Finland)	72186, A. Ylimäki, 1972
29	Barley (Finland)	72187, A. Ylimäki, 1972
30	Wheat (Finland)	72202, A. Ylimäki, 1972
31	Wheat (Finland)	72305, A. Ylimäki, 1972
32	Wheat seed (Israel)	022205, A. Z. Joffe, 1975
33	Wheat stem-base (Australia)	1252, R. L. Dodman, 1975
34	Wheat root (Japan)	SUF 570, T. Matuo, 1960
35	Wheat root (USA)	SUF 995, J. Cook, 1966

a = Abbreviations: DSM-IMB, Deutschen Sammlung für Mikroorganismen am Institut für Mykologie der Biologischen Bundesanstalt, Berlin—Dahlem; CBS, Centraalbureau voor Schimmelcultures, Baarn, Netherlands

of *0.001% bromphenol blue* as a marker dye. The samples passed through the upper gel at *1 mA/tube* and *2mA/tube* in the lower gel until the bromphenol blue marker dye front reached a point 3.5 cm from the stacking-separating gel interface. The gels were immediately removed from the glass tubes and then placed into the appropriate presoak or stain solution. After staining all gels were washed in

Table 4

Fusarium graminearum isolates and their sources

Isolate number	Origin	Source[a]
1	Corn root (Martonvásár, Hungary)	Á. Szécsi, 1971
2	Corn root (Martonvásár, Hungary)	Á. Szécsi, 1931
3	Corn root (Martonvásár, Hungary)	Á. Szécsi, 1971
4	Corn root (Martonvásár, Hungary)	Á. Szécsi, 1971
5	Corn root (Martonvásár, Hungary)	Á. Szécsi, 1971
6	Corn root (Martonvásár, Hungary)	Á. Szécsi, 1971
7	Corn root (Martonvásár, Hungary)	Á. Szécsi, 1971
8	Corn stalk (Martonvásár, Hungary)	Á. Szécsi, 1971
9	Corn stalk (Martonvásár, Hungary)	Á. Szécsi, 1971
10	Corn stalk (Martonvásár, Hungary)	Á. Szécsi, 1968
11	Corn stalk (Martonvásár, Hungary)	Á. Szécsi, 1968
12	Corn cob (Martonvásár, Hungary)	Á. Szécsi, 1967
13	Sorghum stalk (Martonvásár, Hungary)	Á. Szécsi, 1966
14	Corn root (Bóly, Hungary)	Á. Szécsi, 1970
15	Corn root (Bóly, Hungary)	Á. Szécsi, 1968
16	Corn stalk (Martonvásár, Hungary)	Á. Szécsi, 1968
17	Corn root (Baja, Hungary)	Á. Szécsi, 1971
18	Corn stalk (Baja, Hungary)	Á. Szécsi, 1971
19	Corn root (Szeged, Hungary)	Á. Szécsi, 1972
20	Corn stalk (Szeged, Hungary)	Á. Szécsi, 1972
21	Hemp stalk (Bátmonostoros, Hungary)	Á. Szécsi, 1966
22	Wheat root (Szabadbattyán, Hungary)	Á. Szécsi, 1970
23	Barley seed (England)	DSM-IMB 9864, Taylor, 1963
24	Corn stalk (German Fed. Rep.)	DSM-IMB 10948, Schneider, 1968
25	Wheat seed (Bulgaria)	DSM-IMB 11802, Gerlach, 1971
26	Corn (Netherlands)	CBS 166.57
27	Wheat (Netherlands)	CBS 389.62
28	Barley (Finland)	70106, A. Ylimäki, 1970
29	Feed mix (Finland)	7137, A. Ylimäki, 1971
30	Oat (Finland)	72235, A. Ylimäki, 1972
31	Wheat (Finland)	72323, A. Ylimäki, 1972
32	Corn seed (Israel)	022016, A. Z. Joffe, 1975
33	Wheat stem-base (Australia)	1250, R. L. Dodman, 1975
34	Corn root (Japan)	SUF 555, T. Matuo, 1960
35	Wheat ear (Japan)	SUF 1021, T. Matuo, 1969

a = Abbreviations: DSM-IMB, Deutschen Sammlung für Mikroorganismen am Institut für Mykologie der Biologischen Bundesanstalt, Berlin—Dahlem; CBS, Centraalbureau voor Schimmelcultures, Baarn, Netherlands

water and stored in *7% acetic acid.* For esterase at least two separate electrophoretic runs were carried out on a mycelial extract of each isolate.

Detection of esterase activity of gels. The gels were placed in *0.1 M phosphate buffer (pH 6.5)* for 15 minutes at 4°C and then transferred to a staining solution containing *30 mg alpha naphthyl acetate* (Chemapol) dissolved in *2 ml 50%*

acetone, 100 mg Fast Blue RR 12630 (Gurr Ltd.) and *90 ml* of *0.1 M phosphate buffer (pH 6.5)*: incubated 1 hr at room temperature (REDDY and STAHMANN, 1972; NEALSON and GARBER, 1967).

Calculation and identification of R_f values of esterase patterns. The resulting patterns (the gels were stained for esterase) were photographed and analyzed with a *Chromoscan* (Joyce Loebl Ltd.) densitometer. The position of each esterase band was recorded as R_f value, which was calculated as the ratio of the distance migrated by the band in relation to the bromphenol blue dye front. Peaks on the densitometer traces were directly matched with bands in the gel. The R_f values were calculated from the densitometric graph paper profile of each isolate. It was essential to establish for the investigation of the difference, respectively the similarity between *F. culmorum* and *F. graminearum*, which R_f values are corresponding and which are different within the two species and between the two species. We employed for the identification of R_f values a *graphical method.* We drew the R_f values of each isolates on the graph paper stripe which had a division from 0 to 1. Each pattern stripes was compared with one another visually, so that the marked R_f values should as far as possible cover each other. In this way on the basis of R_f values (patterns), also taken numerical R_f values into consideration, we could identify gradually the single R_f value within the species and between the species.

Statistical analysis

We employed simpler statistical analysis instead of the models of numerical taxonomy (cluster analysis, discriminance analysis etc.) because of absence of computer capacity.

Binary contingency tables. The all R_f values were placed in suitable groups for 15 esterase bands on the basis of R_f identification. We constructed one binary contigency table for each of the two species by the aid of these band groups (Table 5 and 6). The lines represent the patterns of single isolates, while the columns represent the R_f values of esterase bands in the table. A *binary function* (n_{ij}) defined for the cells of table so that: $n_{ij} = 1$ if the given R_{f_j} is present, and $n_{ij} = 0$, if the given R_{f_j} is absent in the i^{th} pattern. The S_1 is the *column frequency* of the ones (1), while the S_0 is the column frequency of the zeros (0) in the table. The $S_1\%$ is the *percentage of the frequency values* of the single esterase bands regarding all isolates of species. We calcuated the *difference of S_1 percentages* $(S_1\%)$ attached to each equivalent R_f value of the two species, which marked $D\%$. The $D\%$ is the *difference of frequency values percentage* between the two species, which value is characteristic of the given esterase band. The $D\%$ value represented in the diagram with regard to the *avarage R_f values* of 70 isolates (Figure 6).

X^2-statistics. We employed chi-squared test for establishing if there exists a difference between *F. culmorum* and *F. graminearum* and what kind of significance probability in respect of *frequency values* (S_1) of a single band. We made *2X2 contingency table* in order to investigate this (Table 1). We investigated with the X^2-statistic if a : b and c : d frequency distributions derived from the equivalent statistical population, namely the two species differ from each other signif-

Table 5

Binary contingency table of esterase patterns of *Fusarium culmorum*

Isolate number	1.	2.	3.	4.	5.	6.	7.
1.	1	1	1	0	0	1	1
2.	1	1	1	1	0	1	1
3.	0	1	1	0	0	1	1
4.	1	1	1	0	0	1	1
5.	0	1	1	0	0	1	1
6.	0	1	1	0	0	1	1
7.	1	1	1	0	0	1	1
8.	1	1	1	0	0	1	1
9.	0	1	1	0	0	1	1
10.	0	0	1	0	0	1	1
11.	1	1	1	0	1	1	1
12.	0	0	0	0	1	1	1
13.	0	0	0	0	1	1	1
14.	0	0	1	1	0	1	1
15.	1	1	0	1	1	1	1
16.	0	0	1	0	0	0	0
17.	0	1	0	1	1	0	1
18.	1	1	1	1	1	1	1
19.	0	0	1	1	1	1	1
20.	0	0	1	1	1	1	1
21.	0	0	1	0	1	0	1
22.	0	1	1	0	0	1	0
23.	0	1	0	1	1	0	1
24.	1	1	1	0	1	0	1
25.	0	1	1	0	0	1	0
26.	0	1	0	1	1	1	0
27.	0	0	1	0	0	1	0
28.	1	1	1	1	0	1	1
29.	0	1	1	1	1	1	1
30.	0	1	1	0	0	1	1
31.	0	0	1	0	0	1	1
32.	0	1	1	1	0	1	1
33.	0	0	1	1	0	1	1
34.	0	1	1	0	0	1	1
35.	0	0	1	0	0	1	0
S_1	10	23	29	13	13	30	29
S_0	25	12	6	22	22	5	6
$S_1\%$	28.5	65.7	82.8	37.1	37.1	85.7	82.8

icantly in respect of their R_{fj} values. The statistical term of application of test is the *expected frequency* must not be smaller than five in a single cell either, therefore it cannot be employed with all R_f value. The obtained results are in the Table 9, where the X_c^2 is the calculated value, the X_t^2 is the tabulated value and the $P\%$ is the significance probability at $df = 1$.

8.	9.	10.	11.	12.	13.	14.	15.
0	1	0	1	0	0	0	0
1	1	0	0	0	0	0	0
0	1	0	0	0	0	0	0
0	1	0	0	0	0	0	0
0	1	0	0	0	0	0	0
0	1	0	0	0	0	0	0
1	1	0	0	0	0	0	0
0	1	0	0	0	0	0	0
0	1	0	0	0	0	0	0
0	1	0	0	0	0	0	0
0	1	0	0	0	0	0	0
0	1	1	0	0	1	0	0
0	1	1	1	0	1	0	0
0	1	0	0	0	1	1	0
0	1	0	1	0	0	1	0
0	1	1	0	0	1	0	0
0	1	1	0	0	1	0	1
0	1	0	1	0	0	0	1
0	1	0	0	0	1	0	0
0	1	0	0	0	0	0	0
0	0	0	1	0	0	0	1
0	1	0	1	0	1	0	0
0	0	1	0	0	1	0	1
0	0	0	1	0	1	0	1
0	1	0	0	0	0	0	0
0	1	0	1	0	1	0	0
0	1	0	0	0	1	0	0
0	1	0	0	0	0	0	0
0	1	1	1	0	0	0	1
0	1	0	0	0	1	0	1
0	1	0	0	0	0	0	0
1	1	0	1	1	1	0	0
1	1	0	0	0	0	0	0
1	0	0	0	0	0	0	0
0	1	1	0	0	1	0	0
5	31	7	10	1	14	2	7
30	4	28	25	34	21	33	28
14.2	88.5	20.0	28.5	2.8	40.0	5.7	20.0

The error of the reviewed two methods is, that they investigate the single esterase band individually, although all R_f values (R_f patterns) of the single isolate should have been taken into consideration at the same time to establishing of real similarity. A computer is needed to this. We employed an other X^2-test (SVÁB, 1973) to eliminate above-mentioned scantiness to some extent, which takes

Table 6

Binary contingency table of esterase patterns of *Fusarium graminearum*

Isolate number	1.	2.	3.	4.	5.	6.	7.
1.	0	0	1	0	0	1	1
2.	1	1	1	0	1	0	1
3.	1	1	1	0	1	1	1
4.	0	1	1	0	0	1	1
5.	0	1	1	0	0	1	1
6.	1	1	1	0	0	1	1
7.	0	1	1	0	0	1	1
8.	1	0	1	0	0	1	1
9.	1	1	1	0	1	1	1
10.	1	1	1	0	1	0	1
11.	1	1	1	0	1	0	1
12.	1	1	1	0	1	0	1
13.	1	0	1	0	1	0	1
14.	1	1	1	0	0	1	1
15.	1	1	1	0	1	0	1
16.	1	1	1	0	1	0	1
17.	1	1	1	0	0	1	1
18.	1	1	1	0	1	0	1
19.	0	1	1	0	0	1	1
20.	1	1	1	0	1	0	1
21.	1	1	1	0	1	0	1
22.	1	1	1	1	1	1	1
23.	1	1	1	0	0	0	1
24.	1	1	1	0	1	0	1
25.	1	1	1	0	1	0	1
26.	1	0	1	0	1	1	0
27.	1	1	1	0	1	0	1
28.	1	0	1	1	1	1	0
29.	1	0	1	1	1	0	1
30.	1	0	1	1	1	0	1
31.	1	1	1	1	1	0	1
32.	1	1	0	1	1	0	1
33.	0	0	0	1	1	0	1
34.	1	0	1	0	1	0	1
35.	1	1	1	1	1	1	1
S_1	29	26	33	8	25	15	33
S_0	6	9	2	27	10	20	2
S_1 %	82.8	74.2	94.2	22.8	71.4	42.8	94.2

at the same time the frequency distribution of esterase band into consideration (Table 2). The X^2-test used for the homogeneity of two frequency distributions with the 15 classes in this case. The a_j and b_j are appropriate S_1 values of two species. The results are presented in the Table 10.

8.	9.	10.	11.	12.	13.	14.	15.
0	1	1	1	0	1	1	1
0	1	1	0	0	0	0	0
0	1	1	0	0	0	0	0
1	1	1	1	0	0	0	1
1	1	1	1	0	0	0	1
1	1	1	1	0	1	0	1
1	4	1	1	0	1	1	1
0	1	1	1	0	1	0	1
0	1	1	0	0	0	0	0
0	1	1	0	0	0	0	0
0	1	1	0	0	0	0	0
1	1	1	0	0	0	0	0
0	1	1	0	0	0	0	0
1	1	1	1	0	1	0	1
0	0	1	0	0	0	0	0
0	1	1	0	0	0	0	0
0	1	1	1	0	1	1	1
0	1	1	0	0	0	0	0
0	1	1	1	0	0	0	1
0	0	1	0	0	0	0	0
0	1	1	0	0	0	0	0
0	0	1	1	0	0	0	1
1	0	0	1	0	0	1	0
0	1	1	0	0	1	0	1
0	1	1	0	0	0	1	1
0	1	0	0	0	0	0	1
0	1	1	0	0	1	0	1
0	0	0	0	0	0	0	0
0	0	1	0	0	0	0	0
0	1	1	0	0	0	0	0
0	0	1	0	0	0	0	1
1	0	1	0	0	1	1	0
0	0	1	0	0	1	1	0
0	1	1	0	0	0	0	0
0	1	1	0	0	1	0	1
8	27	32	11	0	11	7	16
27	8	3	24	35	24	28	19
22.8	77.1	91.4	31.4	0.0	31.4	20.0	45.7

Results and Discussion

The esterase patterns of some isolates of *Fusarium culmorum* and *F. graminearum* are shown in Fig. 1 and Fig. 2, respectively Fig. 3 and Fig. 4. The densitometric profile of one isolate of *F. graminearum* is demonstrated in Fig. 5. The R_f values of 15 esterase bands of 35–35 isolates of *F. culmorum* and *F. graminearum* are summarized in Table 7 and Table 8.

On the basis of the results of our investigations the following conclusions can be stated:

1. We could identify 15 esterase bands which had different R_f values in *F. culmorum* and in *F. graminearum* species. The 15 esterase bands occurred in both *F. culmorum* and *F. graminearum*, but with different frequency. We could not demonstrate species specific esterase band, therefore the esterase pattern method is not suitable in practice to separate the two species from one another.

2. We attempted to establish the degree of the difference, respectively the similarity between the two species on the basis of frequency data of esterase bands with the suitable statistical methods. We characterized the degree of the difference and the similarity between certain bands of species separately with *D% values* (Fig. 6). The greater difference was between the bands 1, 5, 6, 10 and 15

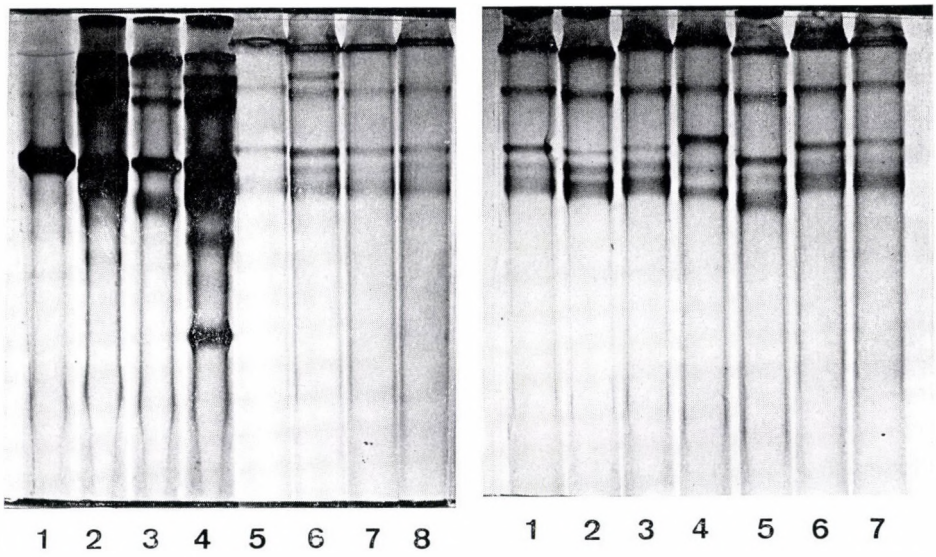

Fig. 1. Esterase patterns of *Fusarium culmorum* isolates. 1. isolate number 22; 2. isolate number 23; 3. isolate number 24; 4. isolate number 25; 5. isolate number 1; 6. isolate number 2; 7. isolate number 7; 8. isolate number 9

Fig. 2. Esterase patterns of *Fusarium culmorum* isolates. 1. isolate number 4; 2. isolate number 3; 3. isolate number 5; 4. isolate number 6; 5. isolate number 8; 6. isolate number 10; 7. isolate number 11

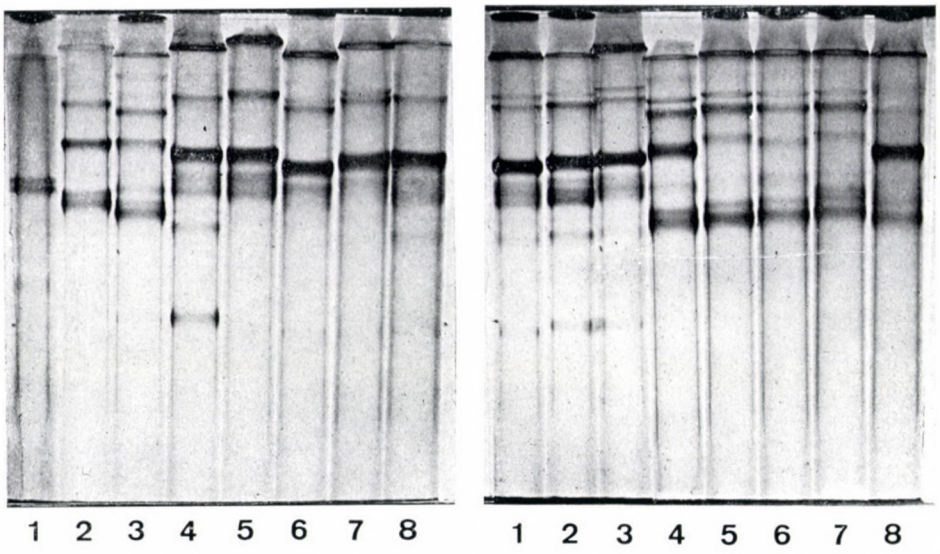

Fig. 3. Esterase patterns of *Fusarium graminearum* isolates. 1. isolate number 23; 2. isolate number 24; 3. isolate number 25; 4. isolate number 1; 5. isolate number 8; 6. isolate number 17; 7. isolate number 19; 8. isolate number 14

Fig. 4. Esterase patterns of *Fusarium graminearum* isolates. 1. isolate number 2; 2. isolate number 3; 3. isolate number 4; 4. isolate number 9; 5. isolate number 10; 6. isolate number 21; 7. isolate number 12; 8. isolate number 13

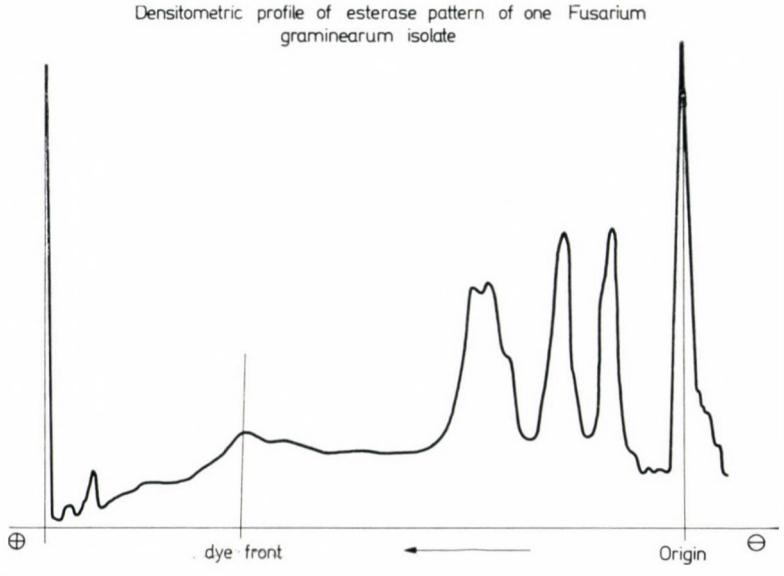

Fig. 5. Densitometric profile of esterase pattern of one *Fusarium graminearum* isolate

Table 7

R_f-value of esterase bands of *Fusarium culmorum*

Isolate number	1.	2.	3.	4.	5.	6.	7.
1.	0.029	0.085	0.134	—	—	0 305	0 360
2.	0.031	0.104	0.129	0.199	—	0 300	0 351
3.	—	0.099	0.125	—	—	0.290	0 339
4.	0.038	0.050	0.122	—	—	0.286	0 340
5.	—	0.110	0.133	—	—	0.292	0.344
6.	—	0.107	0.126	—	—	0.266	0.346
7.	0.035	0.102	0.131	—	—	0.291	0.342
8.	0.044	0.107	0.126	—	—	0.295	0.346
9.	—	0.103	0.125	—	—	0.292	0.345
10.	—	—	0.148	—	—	0.313	0.363
11.	0.068	0.092	0.133	—	0.211	0.300	0.365
12.	—	—	—	—	0.258	0.293	0.384
13.	—	—	—	—	0.251	0.287	0.372
14.	—	—	0.118	0.191	—	0.282	0.344
15.	0.069	0.095	—	0.164	0.208	0.287	0.343
16.	—	—	0.137	—	—	—	—
17.	—	0.109	—	0.184	0.237	—	0.333
18.	0.111	0.139	0.181	0.234	0.279	0.314	0.377
19.	—	—	0.124	0.184	0.224	0.285	0.348
20.	—	—	0.120	0.186	0.229	0.296	0.349
21.	—	—	0.127	—	0.226	—	0.379
22.	—	0.086	0.120	—	—	0.310	—
23.	—	0.098	—	0.169	0.222	—	0.353
24.	0.072	0.101	0.137	—	0.239	—	0.367
25.	—	0.076	0.121	—	—	0.312	—
26.	—	0.094	—	0.208	0.272	0.306	—
27.	—	—	0.132	—	—	0.308	—
28.	0.055	0.079	0.125	0.190	—	0.270	0.361
29.	—	0.086	0.129	0.162	0.195	0.268	0.347
30.	—	0.105	0.134	—	—	0.277	0.364
31.	—	—	0.121	—	—	0.278	0.331
32.	—	0.107	0.134	0.203	—	0.273	0.368
33.	—	—	0.122	0.189	—	0.289	0 342
34.	—	0.089	0.133	—	—	0.263	0.373
35.	—	—	0.132	—	—	0.270	—

8.	9.	10.	11.	12.	13.	14.	15.
—	0.409	—	0.501	—	—	—	—
0.382	0.408	—	—	—	—	—	—
—	0.386	—	—	—	—	—	—
—	0.393	—	—	—	—	—	—
—	0.392	—	—	—	—	—	—
—	0.406	—	—	—	—	—	—
0.378	0.400	—	—	—	—	—	—
—	0.400	—	—	—	—	—	—
—	0.402	—	—	—	—	—	—
—	0.414	—	—	—	—	—	—
—	0.412	—	—	—	—	—	—
—	0.419	0.489	—	—	0.632	—	—
—	0.407	0.482	0.553	—	0.620	—	—
—	0.383	—	—	—	0.630	0.724	—
—	0.422	—	0.531	—	—	0.706	—
—	0.390	0.489	—	—	0.591	—	—
—	0.397	0.489	—	—	0.602	—	0.790
—	0.422	—	0.577	—	—	—	0.758
—	0.402	—	—	—	0.644	—	—
—	0.396	—	—	—	—	—	—
—	—	—	0.576	—	—	—	0.759
—	0.413	—	0.531	—	0.658	—	—
—	—	0.480	—	—	0.604	—	0.819
—	—	—	0.528	—	0.646	—	0.806
—	0.430	—	—	—	—	—	—
—	0.424	—	0.582	—	0.643	—	—
—	0.427	—	—	—	0.644	—	—
—	0.423	—	—	—	—	—	—
—	0.433	0.473	0.533	—	—	—	0.834
—	0.431	—	—	—	0.654	—	0.747
—	0.429	—	—	—	—	—	—
0.385	0.422	—	0.519	0.571	0.638	—	—
0.382	0.408	—	—	—	—	—	—
0.397	—	—	—	—	—	—	—
—	0.412	0.472	—	—	0.604	—	—

Table 8

R_f-value of esterase bands of *Fusarium graminearum*

Isolate number	1.	2.	3.	4.	5.	6.	7.
1.	—	—	0.153	—	—	0.313	0.387
2.	0.083	0.118	0.166	—	0.258	—	0.389
3.	0.074	0.123	0.165	—	0.259	0.331	0.392
4.	—	0.114	0.147	—	—	0.313	0.372
5.	—	0.117	0.143	—	—	0.317	0.371
6.	0.080	0.113	0.146	—	—	0.313	0.373
7.	—	0.119	0.150	—	—	0.321	0.378
8.	0.089	—	0.156	—	—	0.321	0.385
9.	0.080	0.135	0.177	—	0.254	0.322	0.390
10.	0.061	0.122	0.163	—	0.273	—	0.375
11.	0.060	0.112	0.150	—	0.230	—	0.346
12.	0.059	0.119	0.155	—	0.241	—	0.357
13.	0.102	—	0.169	—	0.254	—	0.381
14.	0.083	0.115	0.146	—	—	0.316	0.370
15.	0.067	0.128	0.169	—	0.291	—	0.376
16.	0.061	0.119	0.161	—	0.280	—	0.367
17.	0.086	0.127	0.150	—	—	0.316	0.373
18.	0.068	0.123	0.162	—	0.246	—	0.366
19.	—	0.128	0.147	—	—	0.311	0.369
20.	0.065	0.125	0.164	—	0.263	—	0.398
21.	0.064	0.114	0.158	—	0.257	—	0.365
22.	0.076	0.099	0.127	0.179	0.224	0.307	0.371
23.	0.038	0.069	0.118	—	—	—	0.365
24.	0.059	0.126	0.168	—	0.287	—	0.379
25.	0.058	0.120	0.165	—	0.255	—	0.389
26.	0.053	—	0.168	—	0.242	0.306	—
27.	0.049	0.109	0.137	—	0.243	—	0.367
28.	0.064	—	0.147	0.198	0.274	0.332	—
29.	0.042	—	0.132	0.185	0.264	—	0.378
30.	0.052	—	0.140	0.173	0.293	—	0.349
31.	0.059	0.112	0.145	0.192	0.268	—	0.370
32.	0.035	0.117	—	0.195	0.283	—	0.340
33.	—	—	—	0.180	0.291	—	0.364
34.	0.066	—	0.148	—	0.244	—	0.369
35.	0.026	0.052	0.102	0.125	0.258	0.314	0.357

8.	9.	10.	11.	12.	13.	14.	15.
—	0.415	0.476	0.520	—	0.632	0.697	0.779
—	0.428	0.463	—	—	—	—	—
—	0.431	0.470	—	—	—	—	—
0.392	0.421	0.480	0.526	—	—	—	0.820
0.394	0.421	0.478	0.521	—	—	—	0.779
0.403	0.416	0.476	0.526	—	0.633	—	0.783
0.406	0.430	0.488	0.536	—	0.648	0.716	0.795
—	0.423	0.484	0.563	—	0.646	—	0.773
—	0.445	0.467	—	—	—	—	—
—	0.444	0.475	—	—	—	—	—
—	0.419	0.452	—	—	—	—	—
0.407	0.427	0.463	—	—	—	—	—
—	0.433	0.477	—	—	—	—	—
0.396	0.415	0.460	0.543	—	0.613	—	0.782
—	—	0.457	—	—	—	—	—
—	0.429	0.464	—	—	—	—	—
—	0.415	0.472	0.517	—	0.623	0.686	0.769
—	0.425	0.464	—	—	—	—	—
—	0.408	0.475	0.517	—	—	—	0.765
—	—	0.457	—	—	—	—	—
—	0.432	0.466	—	—	—	—	—
—	—	0.461	0.554	—	—	—	0.766
0.386	—	—	0.519	—	—	0.668	—
—	0.438	0.463	—	—	0.645	—	0.793
—	0.427	0.455	—	—	—	0.699	0.768
—	0.424	—	—	—	—	—	0.771
—	0.416	0.452	—	—	0.632	—	0.752
—	—	—	—	—	—	—	—
—	—	0.472	—	—	—	—	—
—	0.437	0.467	—	—	—	—	—
—	—	0.456	—	—	—	—	0.808
0.382	—	0.450	—	—	0.613	0.712	—
—	—	0.475	—	—	0.614	0.694	—
—	0.406	0.469	—	—	—	—	—
—	0.420	0.476	—	—	0.596	—	0.754

(Fig. 6). The D% values of these bands are above the *average of D% values* ($\overline{D\%}$), the value of which is *21.5*. Accordingly the *average similarity* ($\overline{S\%}$) between the two species is *79.5*. At the bands where we could not employ the X^2-test the D% value was small, namely the similarity was great.

3. The X^2-statistic was employed on the 2X2 contingency tables confirmed the above-mentioned results, because it was applicable in the case of the greatest D% values. We obtained a strong significant difference at $P = 0.1$ *probability*

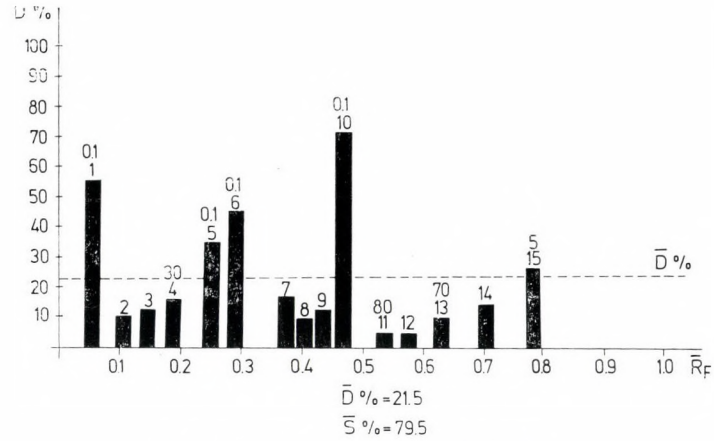

Fig. 6. (Interpretation is in the text)

Table 9

(interpretation is in the text)

Esterase band	1.	4.	5.	6.	10.	11.	13.	15.
X_c^2	42.102	1.258	12.206	20.503	71.306	0.0818	0.1587	6.286
X_t^2	10.827	1.074	10.827	10.827	10.827	0.0642	0.148	3.841
P %	0.1	30	0.1	0.1	0.1	80	70	5

level in case of band 1, 5, 6, 10 (Fig. 6 and Table 9). The difference was at the band 4(P = 30%), at the band 11(P = 80%), and at the band 13(P = 70%) statistically these are not significant (Fig. 6).

4. The X^2-test presented in case of df = 14 at P = 0.1 probability level a strong significant difference between the two species on the basis of the frequency distributions of esterase bands (Table 10). The degree of this difference was expressed the *average of D% values* ($\overline{D\%}$): *21.5.*

Table 10

(interpretation is in the text)

df = 14
$X^2_{calculated}$ = 38.670
$X^2_{tabulated}$ = 36.123
P = 0.001 (0.1%)

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Studies on Powdery Mildews of Cucurbits

II. Life cycle and epidemiology of *Erysiphe cichoracearum* and *Sphaerotheca fuliginea*

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Parallel investigations were carried out on the two pathogens of cucurbitaceous plants, *E. cichoracearum* and *S. fuliginea*. The symptoms and the life cycles of the two mildew species are similar. However, their epidemiology is influenced in a different way by the two most important environmental factors: temperature and humidity. *E. cichoracearum* can germinate and develop between larger temperature intervals than *S. fuliginea*, and the conidia of the former species are able to germinate at lower humidity (94% RH). Moreover, dry conditions are favourable for the development and sporulation of *E. cichoracearum*. The other species, *S. fuliginea*, however is adapted to higher humidity; the conidia are able to germinate only in saturated atmosphere, but infection as well as sporulation occur in both high and lower humidity.

In earlier papers (SZ. NAGY, 1970; 1972) it was reported, that the powdery mildew disease of cucurbits is caused in Hungary by *Sphaerotheca fuliginea* and an *Erysiphe* sp. not identified fully that time. On the basis of the recent investigations the name *E. polyphaga* is not valid (SZ. NAGY, 1975). Therefore the other pathogen causing powdery mildew on cucurbits is *E. cichoracearum*.

The two pathogens cause similar symptoms and their distribution is similar in Hungary. Depending on the weather and cultural conditions, however, one or the other species becomes dominant. It may be supposed, that their ecological requirements are different.

The life cycle of the powdery mildew fungi was divided by BLUMER (1967) into four stages: 1. germination of conidia, 2. infection, 3. sporulation, 4. formation of cleistothecia.

The sporulation of *E. cichoracearum* on squash was studied by REUVENI, COHEN and ROTEM (1971), the biology of *S. fuliginea* on cucumber by TAFRADSHISKY (1963) and the epidemiological pattern of the latter one on squash by REUVENI and ROTEM (1974). Parallel studies on these two pathogens are not known.

The object of this study was to compare the life cycle and biology of *E. cichoracearum* and *S. fuliginea*.

Material and Methods

The life cycles of the two pathogens were studied by means of leaf disc method in laboratory (Sz. Nagy, 1972).

Epidemiology was investigated under laboratory, glasshouse and field conditions. The effect of the main environmental factors (temperature and humidity) was tested in the main stages of the life cycle. The tests of the effect of temperature on germination of the conidia were carried out on glass-slides under various temperature in saturated atmosphere, and the effect of humidity was studied at $22 \pm 1°C$ above the solutions of various salts.

The development of infection and sporulation were studied on leaf discs, and on potted and field plants.

Results

Process of the development

The life cycles of the two fungi studied on cucumber leaf discs and registered under a microscope are similar. Germination of the conidia dusted on the leaf discs begins in the covered Petri dishes, under saturated atmosphere within 24 hours. Germ tubes of the conidia stay affixed by appressoria. Growth of the hypha begins after formation of haustoria. The first chains of the conidia are born on the mycelia on the fifth day after inoculation. There are born 4–5 conidia each day (Euoidium-type). On places free from wind these conidia stay on the conidio-

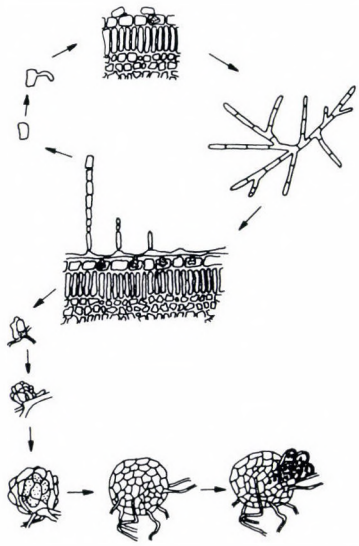

Fig. 1. Life cycle of *Erysiphe cichoracearum*

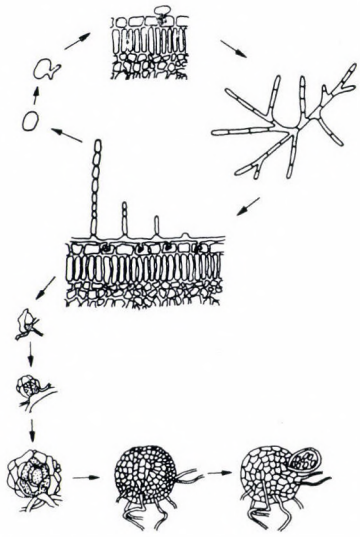

Fig. 2. Life cycle of *Sphaerotheca fuliginea*

phores for days. Formation of the conidia is subsequent on the conidiophores. Chains from 10–20 conidia were often observed. These ones on the end of the chains older than three days are shrivelled. Sometimes there are 8–10 shrivelled conidia on the end of a chain (Figs 1 and 2).

Perfect states of these fungi were not observed on the leaf discs. However, in the glasshouse cleistothecia of *S. fuliginea* were formed on cucumber. And the cleistothecia of the both of the pathogens were observed under field conditions.

Effect of temperature on germination of conidia

In course of parallel tests of the two pathogens it was shown, that in saturated atmosphere the conidia of *E. cichoracearum* can germinate better and between larger temperature intervals than same ones of *S. fuliginea* can.

Germination optimum of *E. cichoracearum* is about 25°C (Table 1), the minimum is 10–15°C, the maximum is 30°C. The optimum of *S. fuliginea* is 22°C, the minimum is 20°C, the maximum is 30°C. Below 10 and 20°C and above 30°C at *E. cichoracearum* and *S. fuliginea*, respectively, germinating conidia only rarely were to be found.

Table 1

Effect of temperature on germination of conidia of *Erysiphe cichoracearum* and *Sphaerotheca fuliginea*

Temperature °C	Percentage germination of conidia	
	Erysiphe cichoracearum	*Sphaerotheca fuliginea*
10±1	in traces	in traces
15±1	5	in traces
18±1	11	in traces
20±1	20	2
22±1	25	10
25±1	29	8
27±1	23	5
30±1	10	2
35±1	in traces	in traces

The temperature has an influence also on the turgor condition of the conidia. Above optimum germination temperature – with increasing of temperature – rises the number of the shrivelled conidia. Below 15 °C no shrivelled conidia were found on glass-slides even after several days.

Effect of relative humidity on germination of conidia

Relative humidity affects the germination of conidia of the two pathogens in different ways, too. The (Table 2) conidia of *S. fuliginea* are able to germinate only in saturated atmosphere, on the other hand the conidia of *E. cichoracearum* are able to germinate even at 94% RH. At 97.5% RH germination was found only

in traces. Below 97.5% RH we could not find germinated conidia on glass-slides.

Table 2

Effect of relative humidity on germination of conidia of *Erysiphe cichoracearum* and *Sphaerotheca fuliginea* on glass-slides

Salt solutions	Relative humidity	Percentage germination of conidia	
		E. cichoracearum	*S. fuliginea*
Dist. H_2O	100	17.3	12.5
4.5% NaCl	97.5	10.8	in traces
9.0% NaCl	94	2.1	0
Saturated KCl	86	0	0
Saturated $NaNO_3$	80	0	0

The relative humidity has an effect on turgor condition of the conidia, too. After 24 hours incubation period at 100% RH 1–2 percent of the conidia was shrivelled. With decrease of humidity the number of the shrivelled conidia increased. No turgid conidia were found below 90% RH. Under similar conditions conidia of *S. fuliginea* shrivelled in higher percentage than those of *E. cichoracearum*. In case of *S. fuliginea* turgid conidia were found only in saturated atmosphere, however, viable conidia of *E. cichoracearum* were found even at 97.5% RH. Below these values only considerably shrivelled conidia were observed.

The parallel studies of these pathogens show, that *S. fuliginea* is more sensitive to moisture than *E. cichoracearum*.

Factors affecting development of infection and sporulation

Laboratory studies

Colonies of both *E. cichoracearum* and *S. fuliginea* developed on cucumber leaf discs between a temperature of 20–25°C. Development was similar at a given daily illumination and in constant darkness. Conidium chains were formed in dark, too.

In the covered Petri dishes, in saturated atmosphere the conidia of *E. cichoracearum* germinated, but no colonies developed. On the other hand colonies of *S. fuliginea* with large numbers of conidia were formed even at 100% humidity in covered Petri dishes.

Glasshouse studies

In the case of glasshouses continuous growing of cucumber in both pathogens were practically found all the year long. In the glasshouse of our institute – where the fluctuation of temperature is comparatively large – *S. fuliginea* on cucumber proved to be more viable than *E. cichoracearum*. Here the plants artificially inoculated with *E. cichoracearum* were repeatedly spontaneously infected by *S. fuliginea*, too.

Field studies

Under field conditions the powdery mildews appear on cucurbits during the first days of July. *E. cichoracearum* was observed on the 5th of July and *S. fuliginea* on the 8th of July for the first time. Both pathogens were found first on cucumber and squash. On melon and watermelon the infection develops later. On melon *E. cichoracearum* was found first on the 21st of July and *S. fuliginea* on the 12nd of August; on watermelon *E. cichoracearum* was found on the 15th of August and *S. fuliginea* on the 3rd of September.

The powdery mildews appear usually after a fall of temperature. Obviously the host plants become more susceptible to powdery mildew infection and the environmental factors become favourable for the pathogens after the rise of temperature, and the infection turns vigorous.

Under rainy, cool weather conditions the same heavy powdery mildew infection was observed as in dry and hot summers. Under spray irrigation cucumber becomes infected in the second half of the summer. Under moisty conditions *S. fuliginea* becomes prevalent.

The first mildew colonies were always observed on close-set plants and on the shaded lower leaves.

Discussion

Previously no simultaneous studies were carried out on the biology and epidemiology of *E. cichoracearum* and *S. fuliginea* on cucurbits. Life cycles of the two pathogens are similar, their ecological requirements however are quite different.

S. fuliginea requires 100% RH for the germination of conidia and during the infection and sporulation tolerates higher moisture content. This observation agrees with the statements of REUVENI and ROTEM (1974). Dry conditions favoured processes of colonization, sporulation and dispersal, while high humidity favoured infection and conidial survival of *S. fuliginea*. However, all these processes occur under conditions of humidity far from their respective optima.

Conidia of *E. cichoracearum* germinate under relative lower moisture conditions, but it needs definitely dry atmosphere for colonization and sporulation. The data of REUVENI, COHEN and ROTEM (1971) about sporulation of *E. cichoracearum* are unacceptable, because of the misidentification of the pathogen (REUVENI and ROTEM, 1974). Identification of the TAFRADSHISKY's mildew is questionable, too.

Acknowledgement

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Ochroconis humicola (Barron et Busch) de Hoog et v. Arx (= *Scolecobasidium humicola* Barron et Busch), a new Record to Hungary

By

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During the isolation of symbiotic *Rhizoctonia* species of various Hungarian orchids a fungus was isolated as a contaminant from the rhizoplane of *Anacamptis pyramidals* (L.) Rich. On the basis of cultural and morphological characteristics it was determined as *Ochroconis humicola* (Barron et Busch) de Hoog et v. Arx. (= *Scolecobasidium humicola* Barron et Busch). This is the first record of this species from Hungary.

Extensive study is being carried on the isolation of symbiotic *Rhizoctonia* species from roots of various Hungarian orchids. Usually several other fungi – contaminants from the rhizoplane – appear on the plates. The majority of these belong to common saprophytic or plant pathogenic groups frequently encountered from soil. One of them, however, *Ochroconis humicola* (Barron et Busch) de Hoog et v. Arx (= Scolecobasidium humicola Barron et Busch) is apparently a new record to Hungary.

Materials and Methods

Roots of *Anacamptis pyramidalis* (L.) Rich. collected near to Dabas (about 40 km SE from Budapest) were washed thoroughly in running water then shaken in 1% solution of benzolsulfonchloramide-Na for 10 min. After several washings in sterile distilled water cross-sections (about 200–500 μ thick) were cut aseptically and placed onto HYH medium of the following composition: honey 20.0 g, yeast extract (Difco) 1.0 g, trace elements solution according to Hoagland 1.0 ml, agar 20.0 g/l. Each dish was inoculated with six sections, then overlayered by 20g/l agar containing 20.0 μg/ml oxytetracycline. Plates were incubated at 28°C and inspected daily for fungal growth.

Results and Discussion

In general, roots of northern temperate orchids are fleshy, fragile organs regularly suffering from minute or more extensive injuries and lesions. Microorganisms living in microscopic cavities of these injuries may survive surface-sterilizing processes and form colonies on the plates. In the present study, two colonies of *Paecilomyces marquandii* (Massee) Hughes, two of a *Fusarium* sp., a sterile mycelium and one colony of *Epicoccum nigrum* Link were observed as contaminants from the rhizoplane. After two weeks of incubation, development of small, dark brown fungal colonies from the rhizodermis of ten inocula were observable. The relatively frequent appearance (about 60% of the total number of contaminants) led to a closer investigation of this fungus. On the basis of its cultural and morphological characteristics it was determined as *Ochroconis humicola* (Barron et Busch) de Hoog et v. Arx (= Scolecobasidium humicola Barron et Busch) – a new record to Hungary.

Fig. 1. Six weeks old colony on HYH medium

The genus *Scolecobasidium* was erected by Abbott (1927) and emended later by Barron and Busch (1962). The type species of the genus, *Scolecobasidium terreum* has Y- or T-shaped conidia, while *Scolecobasidium humicola* produces simple two-celled conidia. De Hoog and von Arx (1973) came to the conclusion, that "the shape of the conidiogenous cells alone cannot be used as the main criterion for the delimitation of *Scolecobasidium;* the shape of the conidia has to be considered as well".

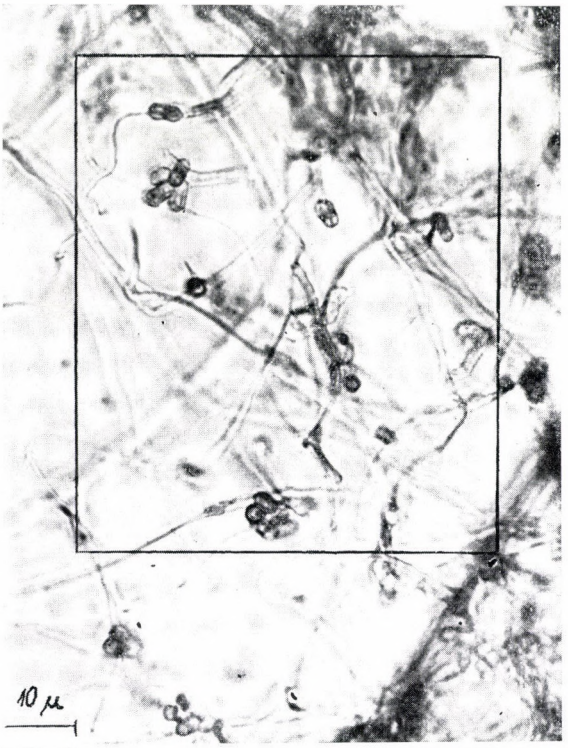
On this basis *S. humicola* has been moved into the new genus *Ochroconis*, erected by de Hoog and von Arx in 1973. At present, Ochroconis is represented by seven species recorded from Australia, Brazil, Ceylon, Egypt, India, Malaya

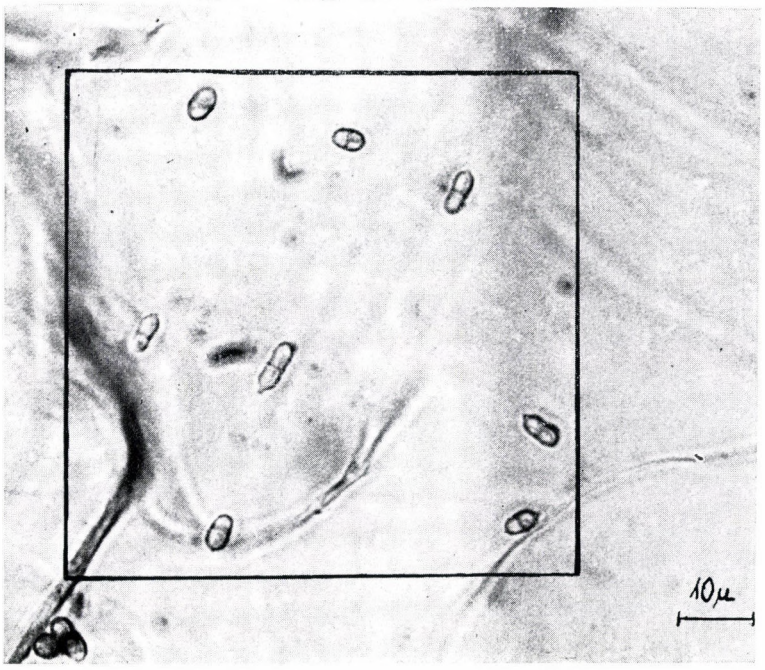
and North America. *O. humicola* was isolated by BARRON and BUSCH in Ontario, USA, from peat soils, cedar bog and a tropical greenhouse of the Ontario Agricultural College at Guelph, Ontario. According to ELLIS (1971) it was isolated from soil and found on *Borassus* palm in India.

The Hungarian habitat shows certain similarities to those of Ontario. The Dabas area has also a marshy character; the *Anacamptis pyramidalis* plant was grown in an atypical *Astragalo-Festucetum rupicolae*, at about 50 m from an *Alnetum hottoniosum* bog. Coenological data of the site are given in Table 1; soil types show mosaic-like distribution reflecting small differences in the elevation. At lower-lying spots typical peat soils can be found.

Fig. 2. Hyphal ropes from the sporulating zones

Ochroconis humicola grows relatively slowly on various media; the giant colony – having a diameter of 70 mm – shown in Fig. 1 is six weeks old. Sporulation of this isolate is rather irregular (or may be absent-cutting of the colony will help in such cases), discrete spots of older colonies may sporulate on hyphal ropes. These areas are darker in color and have a characteristic spiny appearence (Figs 1, 2). Sometimes shining, almost black, smooth patches of secondary growth are observable on some colonies. Conidia (Figs 3, 4) are pale olivaceous brown, finely echinulate, ovoid to short cylindric, uni- or bicellular, occasionally constricted at the septum.





Figs 3—4. Conidia of *Ochroconis huuicola*

Table 1

Coenological data of the Dabas site

Name of species	A—D
Achillea pannonica (Scheele 1844)	+
Anacamptis pyramidalis (L. 1753) Rich. 1818	1
Arabis hirsuta (L. 1753) Scop. 1772	+
Briza media (L. 1753)	+
Campanula sibirica (L. 1753)	+
Cardaria Draba (Resv. 1814)	+
Carex caryophillea (Lafeur. 1785)	+
Centaurea Sadleriana (Janka 1875)	2
Cerastium sp.	+
Chrysanthemum Leucanthemum (L. 1753)	1
Dactylis glomerata (L. 1753)	1
Festuca pseudovina (Hack. ex Wiesb. 1880)	1—2
Festuca rupicola (Heuff. 1858)	3
Filipendula vulgaris (Mönch 1794)	+
Galium verum (L. 1753)	+
Genista tinctoria (L. 1753)	1
Helictotrichon pubescens (Huds. 1763 sub *Avena*) Pilger 1938	+
Hieracium Bauhinii (Schult. ex Bess. 1809)	+
Linum flavum (L. 1753)	1
Linum perenne (L. 1753)	+
Muscari racemosum (L. 1753 sub *Hyacintho*) Mill. 1768	+
Ononis semihircina (Simk. 1879)	1
Orchis coriophora (L. 1753)	1
Orchis militaris (L. 1753)	+
Ornithogalum umbellatum var. angustifolium (Bor. 1847) Gren. et Godr. 1856	+
Phleum phleoides (Karst. 1881)	+
Phragmites communis (Trin. 1820)	+
Pimpinella Saxifraga (L. 1753)	1
Plantago lanceolata (L. 1753)	+
Polygala comosa (Schkuhr 1796)	+
Potentilla heptaphylla (Jusl. 1735)	+
Rhinanthus angustifolius (Gmel. 1806) em. Soó 1968	1—2
Salvia pratensis (L. 1753)	+
Silene nutans (L. 1753)	+
Thesium Lynophyllon (L. 1753)	1
At lower-lying sites:	
Carex distans (L. 1753)	
Festuca pratensis (Huds. 1762)	
Holoschoenus sp.	
Orchis laxiflora (Lam. 1778) ssp. palustris (Jacq. 1786)	
Pastinaca sativa (L. 1753)	
Tetragonolobus maritimus (L. 1753 sub *Loto*) Roth 1788	

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Effect of Internal Leaf Injury on Bacterial Hypersensitivity

By

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Tobacco leaves injected repeatedly with water or rubbed with cotton swabs or carborundum are protected against the hypersensitive reaction induced by *Pseudomonas pisi*. The pattern of bacterial growth was similar to that in leaves protected by prior injection with low concentrations of the bacterium. We suggest that these protective reactions result from a mild nonspecific injury of the tissue.

Tobacco leaves infiltrated with *Pseudomonas tabaci*, a pathogen of tobacco, develop a progressive disease. A rapid cell or tissue death occurs when infiltrated with one of several bacterial species not pathogenic to tobacco. The phenomenon of rapidly developing necrosis of tobacco has been studied extensively since it was suggested that this plant response is another type of hypersensitive reaction, a resistant reaction against plant pathogens (KLEMENT and GOODMAN, 1967).

Factors such as environmental conditions before and after inoculation (HILDEBRAND and RIDDLE, 1971) or number of cells in the inoculum (NOVACKY, ACEDO and GOODMAN, 1973) can limit or modify development of this reaction. In addition, various treatments such as infiltration with heat-killed bacterial cells (LOZANO and SEQUEIRA, 1970) or proteinaceous constituents of bacterial cells (SEQUEIRA, AIST and AISLIE, 1972), cytokinins (NOVACKY, 1972), calcium, strontium and uranium salts (COOK and STALL, 1971), cycloheximide (PINKAS and NOVACKY, 1971) and bovine serum albumin (GÁBORJÁNYI *et al.*) prevent this reaction.

In this report we describe an attempt to relate induction and prevention of bacterially induced rapid necrosis to nonspecific injury of the tissue.

Materials and Methods

Tobacco plants (cv. Samsun NN) were grown in vermiculite irrigated with Hoagland's solution at 24 C day/21 C night with a 14-hour photoperiod and a light intensity of 9×10^3 μ watts/cm^2 (in the laboratory of A. N.) or in soil in

the greenhouse (in the laboratory of P. H.). In both laboratories plants were used in the 10–12 leaf stage.

For the induction of mild injury, leaves were infiltrated with sterile water several times by injection with a hypodermic syringe. Up to 5 injections were applied. All injections following the first were administered after evaporation (disappearance of water soaking) of excess water from the previous injection. Using the same time intervals as the water injections, two other treatments, rubbing with carborundum or cotton swabs (Q Tips) were given.

After treatment, leaves were inoculated with *Pseudomonas pisi* Sackett (ATCC No. 11 055) grown in nutrient broth 24 h at 25 C. The concentration of bacterial cells was adjusted with a densitometer to 5×10^6, 10^7 or 10^8 cells/ml.

Results and Discussion

The development of necrosis caused by *Pseudomonas pisi* ($5 \times 10^6 - 10^7$ cells/ml) was prevented by all treatments used. The challenging inoculation was administered immediately after the last treatment (allowing first a disappearance of water soaking after the water injection treatments) or 24 h, 48 h or 96 h later (Table 1). Development of the protective effect reached its maximum 24 h after the challenging inoculation. At 48 and 96 h the protective capacity declined and islands of confluent necrosis developed (Fig. 1).

The decline of protection coincided with the transfer of protection effects to untreated leaf parts. The transfer of the protective effect was basipetal on treated leaves, but in the whole plants movement was more obvious acropetally to the next upper leaf. Plants held in the dark during the treatments did not show the protective effect.

Other experiments demonstrate that the protective effect against bacterial hypersensitivity can also be induced by a mechanical wounding of the tissue prior to challenging inoculation (Table 1). Bacterial growth in the treated leaves was

Table 1

Effects of water infiltration or mechanical injury on the hypersensitive response

Treatment[a]	Inoculation with *P. pisi*									
	0 hr		24 hr		48 hr		96 hr		1 week	
	5×10^6	10^7	5×10^6	10^7	5×10^6	10^7	5×10^6	10^7	5×10^6	10^7
1. 3× water infiltration	PN	N	P	PN	N	N	N	N		
2. 5× water infiltration	P	P	P	P	P	PN	PN	N	N	N
3. Rubbing (Q Tips) 5×	P	N	P	P	P	P	PN	N	N	N
4. Carborundum 5×	P	N	P	N	P	N				

[a] P = protection, PN = partial necrosis, N = necrosis

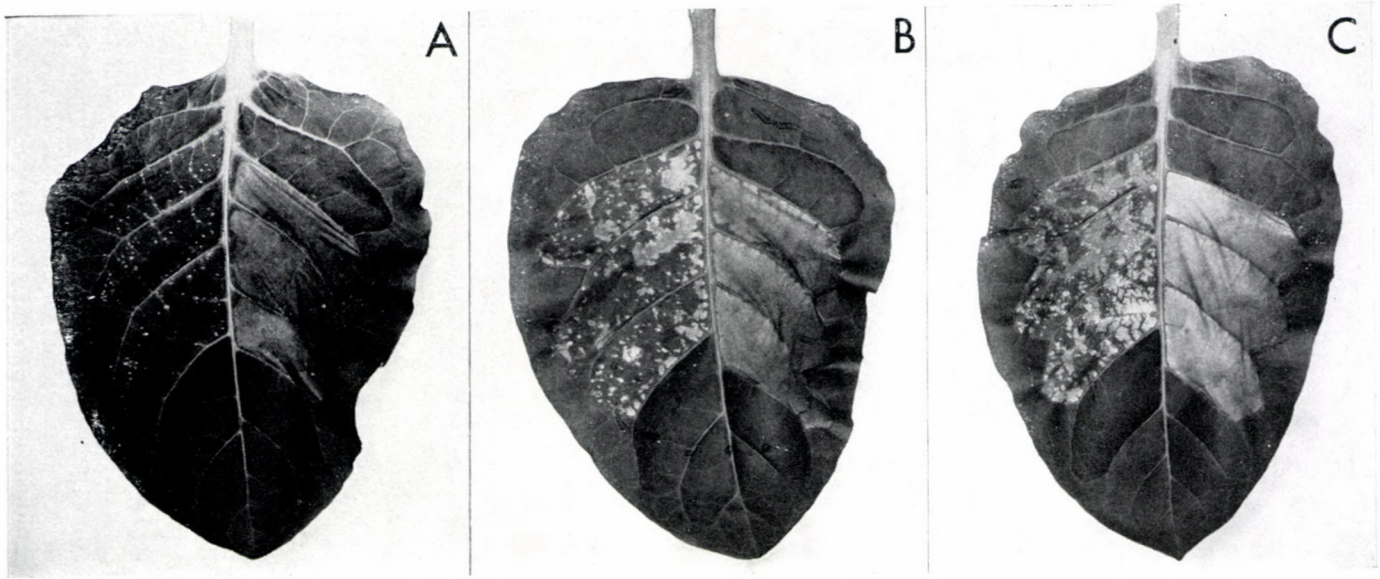

Fig. 1. Tobacco leaves infiltrated with water 5 times prior to inoculation with *P. pisi* (10^7) cells/ml. A. Inoculation after the water evaporated from the previous injection. B. Inoculation 48 h and C. 96 h after the last injection. Right halves are untreated controls

similar to growth of *P. pisi* in tissues protected by low concentrations of the same bacterium (NOVACKY, ACEDO and GOODMAN, 1973) (Fig. 2). Partial protection against 10^8 cells/ml was found in a few experiments.

The character of the leaf damage is unclear, however treated, unchallenged leaves develop mild chlorosis. Recent ultrastructural studies of tobacco leaf tissue inoculated with *P. pisi* (GOODMAN, HUANG and WHITE, 1976) or avirulent *P. solanacearum* (SEQUEIRA, 1976) demonstrate alterations of cell walls and wall surfaces. These alterations are suggested to be involved in localization (immobilization) of incompatible bacteria. Preliminary studies show similar cell wall abnormalities are elicited by treatments used in this study (HANCHEY, PASTALKA and NOVACKY, 1974).

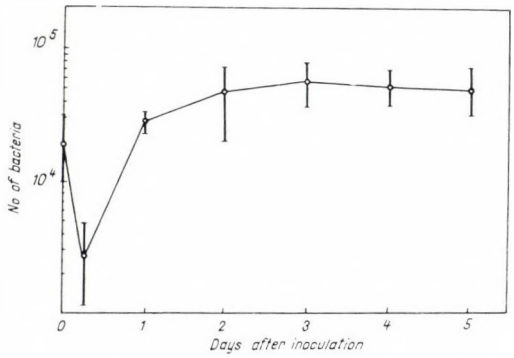

Fig. 2. Growth of *Pseudomonas pisi* in tobacco leaves treated with five consecutive water infiltrations prior to inoculation. Inoculum contained 5×10^6 cells/ml. Number of bacteria recovered from 1 cm² discs is shown at each point

Our observations, however, should not be generalized. SEQUEIRA (personal communication) was unable to repeat the protective effect described here when an incompatible strain of *Pseudomonas solanacearum* (inducing a rapid cell death) was used. Similarly, attempts to repeat these or other treatments in other plant species: cotton (A. N. unpublished) or pepper (COOK, 1975) were unsuccessful.

Our experiments demonstrate that tobacco tolerance to incompatible (nonhost) bacteria is increased with mild internal injury. An injury seems to be a common denominator of various protective treatments. Recently it was reported that ozone injury can induce protection against bacterial hypersensitivity (LUKEZIC *et al.*, 1976). Similarly, low bacterial concentrations (limited injury – TURNER and NOVACKY, 1974) protect against higher bacterial dosages (NOVACKY *et al.*, 1973).

At this point it is interesting to compare protection against bacterial hypersensitivity and protection against injuries caused by heat or ozone. Analogously to protection against bacterial hypersensitivity these injuries also can be suppressed or prevented by low doses of heat or ozone (YARWOOD, 1961; RUNECKLES and

ROSEN, 1974). One can speculate than that the bacterial hypersensitivity is in essence a tissue injury.

The rapid death of cells and tissues as a critical factor localizing the fungal pathogen was recently challenged in two laboratories (KIRÁLY *et al.*, 1972; MAYAMA *et al.*, 1975). In light of these two reports cell death is either a result of some toxic product of the pathogen (KIRÁLY *et al.*, 1972) or a result of stress (MAYAMA *et al.*, 1972). The role of cell and tissue death in the resistance against bacteria and the nature of the toxic factor(s) by which living pathogenic bacteria elicit this reaction await clarification.

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Bacterial Fasciation of *Pelargonium hortorum* in Hungary

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Bacterial fasciation of *Pelargonium hortorum* was found in Hungary in 1972. The symptoms consiste of many short, thick and aborted new shoots or galls occurring near the soil or at the cutting wounds on the affected plants. The pathogenicity of the isolates was proved on sweet peas and geraniums. Pathological and bacteriological tests indicated that the causal organism was *Corynebacterium fascians* (Tilford) Dowson.

During a survay of a greenhouse in December 1972 "fastiation" or "leafy-gall" disease of geranium (*Pelargonium hortorum* (Bailey)) caused by *Corynebacterium fascians* (Tilford) Dowson was found in Budapest. Before 1972, the disease may had existed but until 1972 all attempts on the diagnosis were apparently based on disease symptoms without isolation of the causal bacterium. It is therefore not certain whether *Corynebacterium fascians* or *Agrobacterium tumefaciens* was the cause. Since the disease has not been recorded previously in Hungary this paper describes the symptoms and provides proof of the causal organism. The disease was first reported by BROWN (1927) as occurring near Washington on sweet peas *(Lathyrus odoratus)* and attributed its cause to a week or highly specialized strain of *Bacterium tumefaciens.*

Later on, in America TILFORD (1936) and in Britain LACEY (1936) discovered that this type of fasciation was due to a new Gram-positive bacterium *Phytomonas fascians n. sp.*, now known as *Corynebacterium fascians* (Tilford) Dowson. Since 1936, the disease has been reported from Sweden by LINDFORS (1938), from Denmark by BUCHWALD (1942), from Germany by PAPE (1938) and STARK (1955), from the Sovietunion by VINKALNE (1962). Recently, MAAS-GEESTERANUS *et al.* (1966) investigated the disease and demonstrated in an indirect way that the bacterium caused by itself little harm to *Pelargonium zonale.* In France, FAIVRE-AMIOT (1967) reported the bacterium from several new hosts and stated that the disease has become of economic importance mainly on strawberry and Dahlia.

Materials and Methods

Small pieces from the base of proliferated small shoots were taken and washed in successively in steril water. The pieces were not surface sterilized as the bacterium grows chiefly upon the surface of diseased tissue. Slices were taken from the washed pieces, crushed in steril water, and the suspension inoculated on to agar plates. The most satisfactory medium for isolating the organism was found to be the potato-dextrose agar (PDA) supplemented with 0.1% yeast extract. Isolations on PDA were incubated at 25°C. Growth of the bacterium was slow, 2–3 mm diameter colonies taking 5–6 days to develop. Contaminations were frequent, but at times it was possible to obtain almost pure cultures of the bacterium direct from diseased shoots. After purification tests by repeated plating, single yellow colonies were transferred to agar slopes of PDA or nutrient agar supplemented with yeast extract. For proof pathogenicity sweet pea seeds were surface sterilized with 0.1% mercuric chlorid solution for 15 min, and then washed in several changes of steril water and put to germinate in Petri dishes moistened, sterilized filter paper. Inoculations were made according to TILFORD (1936) by pouring 48 hours old suspension of bacteria over the germinating seeds. The inoculated seeds were transferred to tubes containing sterilized sand or solidified Hoagland's medium. The inoculation of geranium shoots was much more difficult. Numerous attempts were made during several years to reproduce the typical disease symptoms on geranium. At last, the vacuum infiltration method prooved to be successful. The method consisted of immersing hardly rooted young geranium cuttings in a suspension of the bacterium and infiltrating the whole shoot and root by vacuum. The bacterial suspension contained about 10^8 cells/ml. Control plants were handled in the same manner except that they were infiltrated with steril tapwater. The inoculated cuttings were potted in clay pots containing steril soil. The plants were held in normal greenhouse condition and watered daily.

The pathogenicity of the isolates were tested first, on sweet peas and afterwards on geraniums. Four coltures isolated from geraniums and proven pathogenic on sweet peas, and one culture reisolated from diseased geranium were characterized bacteriologically. Morphological and physiological characteristics were examined according to standard methods as recommended by COWAN and STEEL (1965). All tests were performed at 25°C. Fermentation tests were run in a pepton-free basal medium. Bromthymol blue was added to the medium as indicator. Sugars were autoclaved as 10% solutions and added separately to give 0.5 and 1% final concentrations. Gelatin hydrolysis was tested on gelatin agar by FRAZIER's (1926) method after incubation for 7 days. Lecithinase activity was determined on egg yolk agar (MACFARLANE *et al.* (1941)). The method of SIERRA (1957) was used for lipase. Levan production was tested according to LELLIOTT *et al.* (1966).

Results

Symptoms. The host cells of the plant, unlike many other known bacterioses, are not destroyed by the bacterium, but are, rather stimulated and provoked into uncontrolled and mostly irregular cell division. Symptoms of the disease on geranium are rather distinctive and usually quite obvious. Many short, thick and aborted new shoots and galls appear at or below the soil line on affected plants (Fig. 1). Some of these fleshy shoots become fasciated and give rise to misshapen and aborted leaves. Aerial infection may also occur, galls are formed on the main stem which at first sight are indistinguishable from those due *Agrobacterium tumefaciens.* The above ground portions of the aborted shoots and galls are a normal green color similar to the color of the rest of the plant. The underground portions are usually pale yellow. Diseased plants may appear to grow normally, but they

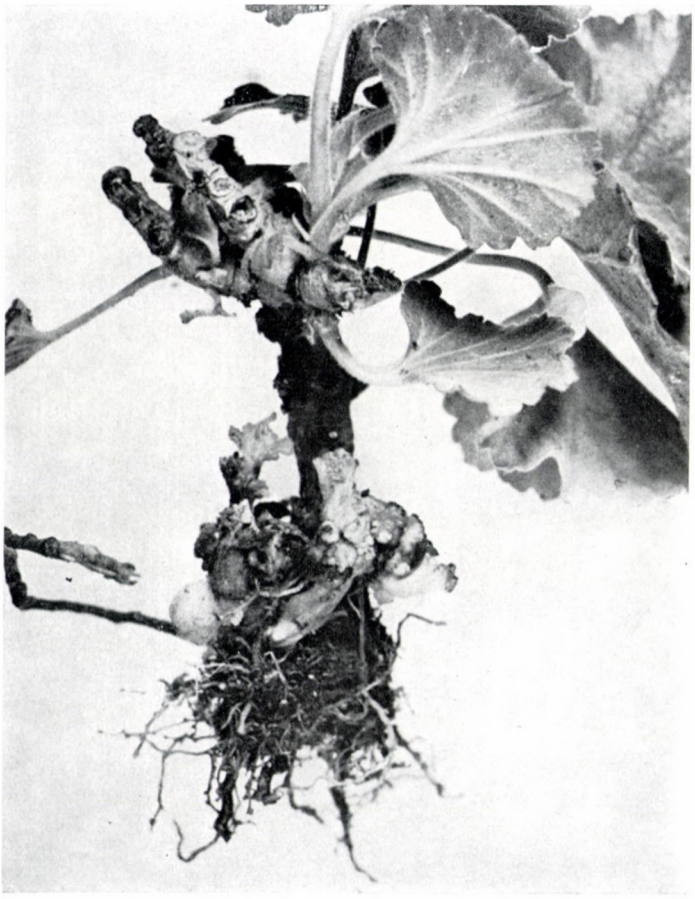

Fig. 1. *Pelargonium hortorum* showing natural infection by *Corynebacterium fascians*

are usually dwarfed and worthless to the market. After the artificial inoculation, the disease symptoms appeared within 3–4 weeks and were similar to the original disease symptoms. However, no more than 20% of the inoculated plants showed the typical symptoms and many of them rotted. Bacteria identical with those used as inoculum were isolated from the new deformed thick leaves of artificially infected plants.

In *Lathyrus odoratus*, the main symptom is characterized by fasciated shoots originate either at the first or cotyledonary node of the stem (Fig. 2). The mass of fasciated growth on old plants resembles a "witches broom". After the inoculation of sweet pea seedlings the proliferated hypocotyl buds proved that the inoculated bacterium was, in fact, *Corynebacterium fascians* (Tilford) Dowson. In that way, within 10–14 days, 80–90% infection was easily attained.

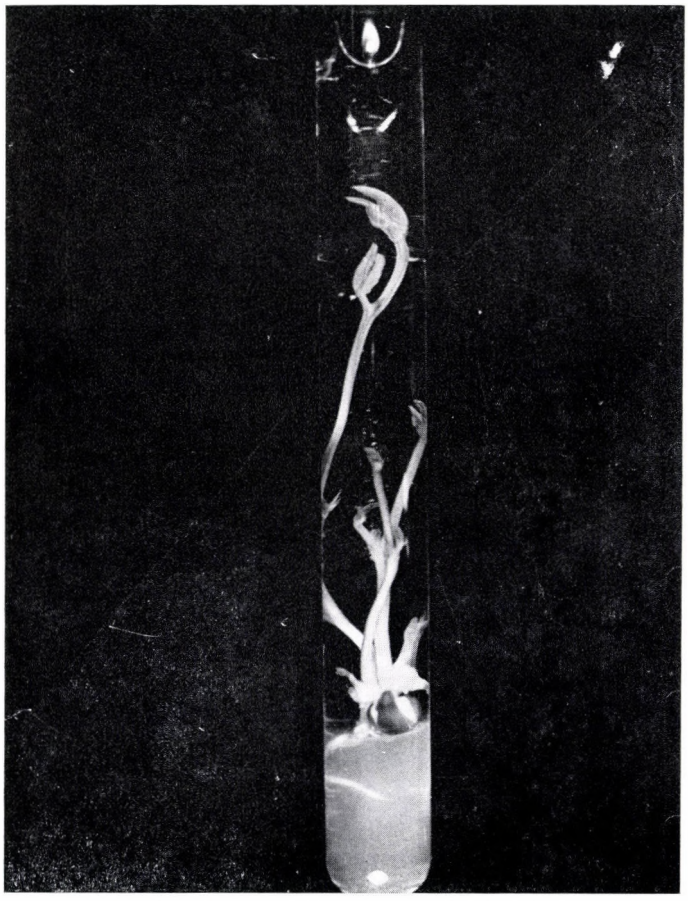

Fig. 2. Pathogenicity test on sweet pea in steril tube

Identification of the organism. The bacteria were non-motile, Gram-positive rods of variable length and width, frequently occurring singly and rarely arranged in characteristic V or X groups. On PDA, colonies were circular, smooth, raised, margin entire and mucoid with yellow pigment. The optimum temperature of growth was in the range of 22–27°C.

Glucose, fructose, lactose, L-arabinose, galactose, maltose, mannose, sucrose, D-xylose, glycerol, mannitol were oxidatively metabolized by all isolates. Growth occurs on raffinose but acid production was not detectable. Rhamnose and salicin were not metabolized. Citrate, lactate and malonate were utilized but utilization of propionate varied according to strain. All isolates gave a negative test for gelatinase, oxidase and indole but a positive one for catalase, urease, lecitinase and levan. Litmus milk was peptonized. No culture hydrolized starch

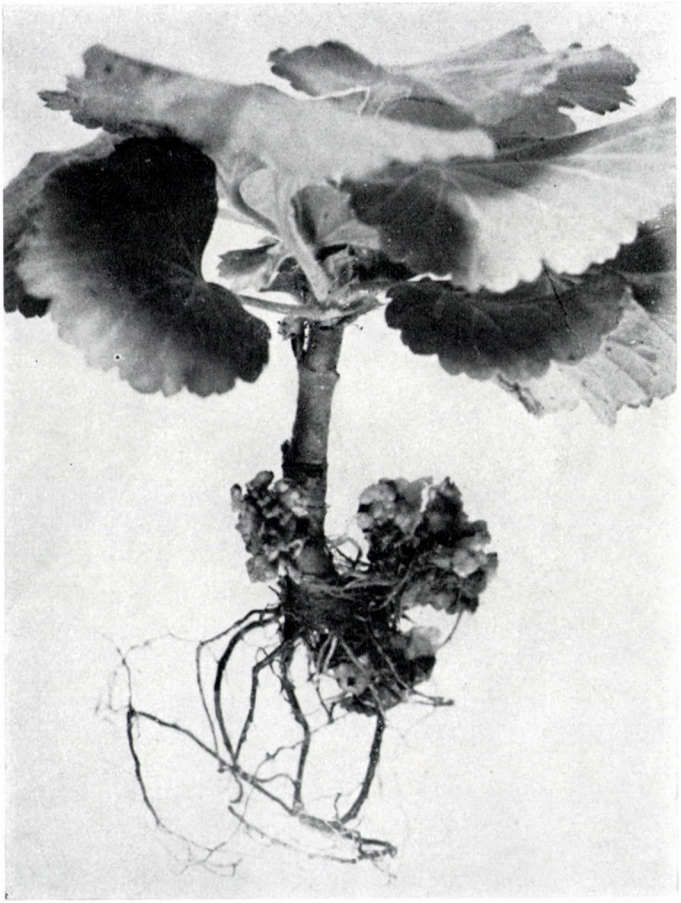

Fig. 3. Bacterial fasciation of geranium caused by artificial inoculation with *Corynebacterium fascians*

and aesculin. Hydrogen sulphide was formed from cysteine hydrochloride, sodium thiosulphate and peptone. Nitrate reduced to nitrites and ammonia was produced from peptone. Tween 80 was slightly hydrolyzed.

Discussion

The symptoms of natural and artificial infections, and the cultural and biochemical reactions of the causal organism show that the Hungarian isolates are similar in all respects to those of *Corynebacterium fascians* (Tilford) Dowson. The characteristic of these isolates are in agreement with those of TILFORD (1936) and MOHANTY (1951). Inoculations with pure cultures of *Corynebacterium*

Fig. 4. Typical fasciation of sweet peas caused by *Corynebacterium fascians*

fascians (Tilford) Dowson obtained from proliferated shoots induced the typical symptoms of the disease on sweet pea (Fig. 4) and geranium (Fig. 3). The reproduction of symptoms on geranium was much more difficult than on sweet pea. The reason for the small proportion of artificial infected geranium is not understood. Other workers too, have found the inoculation of geranium to present some difficulty (FAIVRE-AMIOT, 1967). It is possible that the lower number of successful inoculations were due to some unknown factors. CROSS and PITCHER (1952) reported that the typical cauliflower symptoms on strawberry were reproduced only by a combination of the eelworm and strains of *Corynebacterium fascians* (Tilford) Dowson originally isolated from cauliflower strawberries. So far, there is no evidence that eelworm or any other organism is involved in the infection process of geranium, although it is worth noting that heavily infected plants were obtained from mite-infested greenhouses.

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Ultrastructure of Bacteriophages of *Xanthomonas malvacearum*, the Causal Organism of Bacterial Blight of Cotton

By

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A bacteriophage strain specific to lactose utilizing isolates of *Xanthomonas malvacearum* (E. F. Smith) Dowson, the incitant of bacterial blight of cotton was investigated. The phage was typically like a tadpole with hexagonal head (62.5 nm diameter) and a long flexuous non-contractile tail (145 nm).

Bacteriophages specific to *Xanthomonas malvacearum* (E. F. Smith) Dowson, the incitant of bacterial blight of cotton were isolated in India by SINGH *et al.* (1970). Various properties of these phages are known (SINGH and VERMA, 1974) but not their morphology. In this paper we report the morphology of a bacteriophage specific to lactose utilizing isolates of *X. malvacearum*.

Materials and Methods

Bacteriophages were isolated from phage-enriched-broth culture by differential centrifugation and filtration through bacteriological sintered glass filters by standard procedures (SINGH and VERMA, 1974). These phages were characterized and one isolate (XMP—26), which lysed only lactose utilizing isolates of *X. malvacearum* was selected for electronmicroscopical studies.

High titred phage solutions were subjected to differential centrifugation of alternate low (10,000 rpm) and high speed (30,000 rpm) followed by sucrose density gradient centrifugation (VARMA *et al.*, 1970). The purified phage preparations were sprayed on formvar coated copper grids, stained with 2% neutral phosphotungstic acid (BRADLEY, 1967) or 0.5% uranyl formate and examined in Philips EM—300.

Results and Discussion

The phages were typically like a tadpole with hexagonal head and a long flexuous, non-contractile tail (Fig. 1). The tails are interesting because they were quite long, more than two times the size of the head, and further because these

coiled to form a loop. The average diameter of the head was 62.5 nm and length of the tail 145 nm. In an earlier study ROSBERG and PARRACK (1955) reported that the phages of *X. malvacearum* were spherical (27 nm in diameter) and without distinct tails, although suggestion of a tail was observed in some phage particles. Bacteriophages specific to *X. malvacearum* are known to exist for some time (MASSEY, 1931; LEVEDEVA, 1937), and they have been classified into two groups i.e. group-I lysing only lactose non-utilizing isolates of *X. malvacearum*, and group-II which lysed only lactose utilizing isolates (HAYWARD, 1964; SINGH and

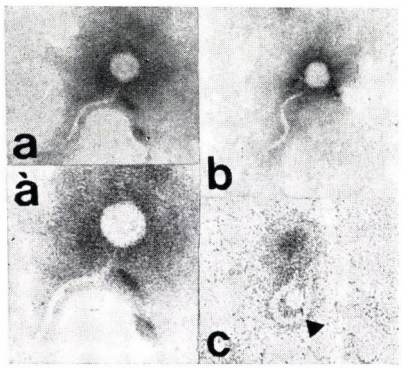

Fig. 1. Electronmicrographs of bacteriophages specific to lactose utilizing isolates of *Xanthomonas malvacearum*, incitant of bacterial blight of cotton; a, b, complete phages stained with PTA showing hexagonal head and flexuous tail (×70,000); á, same as a (×103,500); c, bacteriophage stained with uranyl formate showing coiled tail (arrow): (×103,500)

VERMA, 1974). It is obvious that different kinds of phages attack the same pathogen. It is not known whether ROSEBERG and PARRACK (1955) examined phages of group-I or II. It is possible that morphologically phages of group-II are different from group-I or both the groups have phages of different kinds. This needs further investigations and testing of a larger number of phage isolates.

The present phage resembles clearly in morphology one of the phage isolates (CP_1) of *Xanthomonas citri* which also has hexagonal head (68 nm diameter) and a long (160 nm) flexuous tail (ARAI *et al.*, 1974). The phage isolate CP_2 of *X. xitri* was, however, different. The phage reported here belongs to the group "B" proposed by BRADLEY (1967) or the *Caudaevirus* group (WILDY, 1971) characterized by hexagonal head and a tail, which is flexuous, non-contractile, devoid of any terminal appendage and much longer than the head diameter.

Acknowledgement

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Effect of Ethylene on Potato Tubers Inoculated with *Erwinia carotovora* var. *atroseptica*

By

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Inoculation with *E. atroseptica* significantly increased the CO_2 and ethylene production of tubers. Externally added ethylene suppressed the CO_2 evolvement of the inoculated tubers, but did not influence that of the healthy ones. The resistance of inoculated tubers to *E. atroseptica* was not significantly influenced by ethylene treatment. No considerable change was noticed in the concentration of rishitin and lubimin as a result of ethylene treatment. The phytuberin production of infected tubers was suppressed by ethylene. Phytoalexin production cannot be induced in healthy potato tubers by ethylene treatment only.

Diseased plants were often observed to evolve more ethylene than healthy ones (WILLIAMSON, 1950). This phenomenon is more characteristic of diseases involving necrosis (LUND, 1973; HISLOP *et al.*, 1973; BALÁZS *et al.*, 1969) rather than of compatible host-parasite relationships (ROSS and WILLIAMSON, 1951). In some cases it has been shown that ethylene is generated mainly at the margin of the lesion and very little is produced from the body of the rot, where the host cells are dead (IMASEKI *et al.*, 1968; HISLOP *et al.*, 1973). It has not been ascertained unambiguously whether it is only the host cells which generate the ethylene or the pathogen also contributes. Many fungi, including plant pathogens, are known to evolve ethylene (ILAG and CURTIS, 1968; CHALUTZ and DEVAY, 1969; ILAG, 1972; LYNCH and HARPER, 1974) as are certain bacteria (FREEBAIRN and BUDDENHAGEN, 1964).

Exposure to ethylene affects different physiological phenomenons such as respiration and some parameters which, independent evidences suggest, are connected with disease resistance, e.g. increases in a number of phenolic compounds in a variety of species, increases in some enzymes leading to synthesis of these chemicals (HISLOP *et al.*, 1973) and an increase in lignin biosynthesis (RHODES and WOOLTORTON, 1973). In a number of reports direct connexion is shown between disease resistance and ethylene treatment. According to STAHMANN and co-workers (1966) ethylene may serve as a part of the defense mechanism of plants. They found that ethylene treatment increased the levels of peroxidase and polyphenoloxidase in sweet potato tubers and later it was shown that there was close correlation between the activity of these enzymes and the resistance of sweet potato to *Ceratocystis fimbriata* (WEBER *et al.*, 1967). CHALUTZ and DEVAY (1969) failed to confirm STAHMANN's findings.

CHALUTZ and co-workers (1969) induced production of antifungal isocoumarin by ethylene treatment in carrot root tissue in a concentration as high as found in tissues inoculated with fungus. However very small amount of phytoalexin pisatin was produced in pea-pods treated with ethylene (CHALUTZ and STAHMANN, 1969). It was suggested that ethylene induced some enzymes participating in synthesis of pisatin, e.g. phenyl-alanine-deaminase (HADWIGER, 1968) but not others.

It was shown that ethylene treatment increased polyphenoloxidase but not peroxidase and phenyl-alanine-ammonia-lyase activity in potato tissues (STAHMANN *et al.*, 1966; HYODO and YANG, 1974).

Experiments were carried out to examine the effect of ethylene on the resistance and phytoalexin production of potato tubers inoculated with *Erwinia carotovora* var. *atroseptica*, and measurings were done on CO_2 and ethylene evolvement of tubers.

Materials and Methods

Tubers. Potatoes were grown in Norfolk, England. Tubers of cultivar Desiree *(Solanum tuberosum)* were harvested in September 1973 and 1974. Tubers harvested in 1973 were stored at 3°C, those harvested in 1974 were stored immediately after harvesting at 10°C for two weeks, the remainder of storage was at 3°C. Experiments were carried out in September–October 1974.

Bacterium. Erwinia carotovora var. *atroseptica (E. atroseptica)* strain G120 (LUND and NICHOLLS, 1970; LYON, 1972; BECZNER and LUND, 1975) was used.

Inoculation of tubers. Bacteria were grown on slopes of "Difco" Heart Infusion Agar at 30°C for 24 h. Growth was washed from the slopes with sterile distilled water and suspension was adjusted to contain approximately 10^9 bacteria per ml. The inoculation of tubers was as described by LYON *et al.* (1975) using the shallow wound method. Ten-eleven tubers were inoculated per treatment and control tubers were wounded and treated with sterile water.

Incubation of tubers and the ethylene treatment. The inoculated tubers in two replicas were separately placed in glass chambers through which was maintained a flow (4.2 l/h in the first and 5.2 l/h in the second experiment) of humidified air containing 9–10 and 14–16 ppm ethylene, respectively. Control, sterile water treated tubers were incubated in separate containers. The control experiments were set up similarly but the air supplied did not contain externally added ethylene. The tubers were incubated at 10°C for 16 days. In the experimental containers the CO_2 content was measured daily, the ethylene concentration twice a day. The air flow rate was checked in every third day.

Estimation of CO_2 and ethylene. The ethylene was measured on gas chromatograph set up in the Food Research Institute, Norwich, fitted with a flame ionization detector and a 570 mm by 4 mm i.d. stainless steel column packed with Porapak S (80–100 mesh). The carrier gas was nitrogen, flow rate 48 ml/min, the flow rate

of air was 700 ml/min, that of hydrogene 38 ml/min. The column temperature 20–22°C (isothermal conditions). Air with known ethylene concentration was injected to each detection as standard. Quantitative analyses were carried out by relating the heights of peaks to that of the standard. Ethylene concentration was worked out in $\mu l.kg^{-1}.h^{-1}$.

Production of CO_2 was measured using "Pye" series 104 gas chromatograph fitted with a katharometer detector (FISHWICK and ZMARLICKI, 1970). The katharometer detector and column were maintained at a temperature of 50°C and the carrier gas helium at a flow rate of 50 ml/min. CO_2 was separated on a 610 mm steel column containing 80–100 mesh Porapak Q (Waters Associates Ltd.). The bridge current was 240 mA. Air sample with known CO_2 concentration was used as standard. Quantities of CO_2 were calculated as described for ethylene and given in $ml.kg^{-1}.h^{-1}$. The comparisons of concentrations of CO_2 and ethylene were made by means of *t*-tests.

Measurement of amount of rotting. At the end of the incubation period, rotted tissue was removed by scraping and its amount was determined by weighing each tuber before and after removing the rots. In the control tubers treated with sterile water a small suberized plug of tissue was found. The tissue had not rotted and it could be easily removed. The weight of rot served as an indirect index of resistance, of course in comparison to the other treatment, only. The weights of rots were compared by means of *t*-tests.

Extraction of phytoalexins. The method of extraction of phytoalexins rishitin, phytuberin and lubimin and their estimation and purification on t.l.c. prior to g.l.c. were as described by BECZNER and LUND (1975).

Estimation of phytoalexins by g.l.c. A "Pye" series 104 gas chromatograph fitted with flame ionization detector was used. The glass column (152.5 cm by 0.4 cm i.d.) was packed with 5% OV–17 on "CQ" (siliconized Celite) 85–100 BSS mesh (JJ's chromatography Ltd., King's Lynn, England). Conditions were as follows (LYON, 1972): carrier gas (argon) 45 ml/min and column inlet pressure of 0.56 kg/cm^2, 45 ml/min for hydrogene and 500 ml/min for air, injection temperature 250°C, detector temperature 250°C, column temperature 200°C (isothermal conditions).

The retention times of rishitin, phytuberin and lubimin relative to methyl stearate internal standard (about 17 min) were 0.59, 0.50 and 0.85, respectively. Quantitative estimations were made by relating peak areas (height of peak × width at half height) to that of the internal standard. The average response ratios were for rishitin 0.69, for phytuberin 0.85 and for lubimin 0.72.

Results

Estimation of CO_2 and ethylene. Results for CO_2 production by tubers are given in Table 1. For the quantities of CO_2 produced varied slightly during the incubation time, we considered them as more or less constant values. The differ-

Table 1

CO_2 production by tubers inoculated with *E. atroseptica* ($ml.kg^{-1}.h^{-1}$)

Incubation time (day)	Experiment I		Experiment II			
	Air		Air		+14—16 ppm ethylene	
	Inoculated	Control	Inoculated	Control	Inoculated	Control
1	29.91	26.46	25.02	20.50	28.00	27.50
2	40.38	34.44	25.80	19.74	34.50	31.50
3	39.58	33.60	—	—	33.00	32.50
4	—	—	31.67	21.64	—	—
5	—	—	34.80	22.39	28.50	24.50
6	37.56	24.36	34.02	21.64	29.50	26.50
7	35.54	24.78	39.49	23.91	27.00	24.00
8	42.00	25.62	36.36	20.88	22.00	18.50
9	37.37	23.94	—	—	21.00	17.50
10	42.40	—	—	—	—	—
11	—	—	—	—	—	—
12	—	—	30.89	17.84	21.50	15.50
13	36.35	18.90	28.93	16.70	26.50	20.50
14	42.40	23.10	34.80	20.12	26.00	19.00
15	41.19	21.00	39.88	24.67	28.00	20.50
16	—	—	—	—	27.00	20.00
mean	38.60	25.60	32.87	20.90	27.11	22.92
st. dev.	3.69	4.72	4.73	2.26	3.87	5.13
t-test	7.088***		7.608***		2.365*	
mean			32.87		27.11	
st. dev.			4.73		3.87	
t-test				3.284**		
mean				20.91		22.92
st. dev.				2.26		5.13
t-test					1.202	

Exp. I tubers stored for 1 year at 3°C
Exp. II freshly harvested tubers[3]
— no data

Significance: *P = 5%
**P = 1%
***P = 0.1%

ence in CO_2 production between Erwinia-inoculated and control tubers was strongly significant (P = 0.1%) in both experiments, in case the tubers were incubated in air, only. When ethylene was added to the incubation atmosphere (the CO_2 production was measured in experiment II only) the difference was significant at level of P = 5%. Less CO_2 was produced by Erwinia-inoculated tubers incubated in ethylene than by those incubated in air. The difference was significant at P = 1%. The control (wounded but not inoculated) tubers evolved more or less the same amount of CO_2 during incubation in air as well as in ethylene.

The same method of evaluation was used for ethylene production. The concentration of ethylene was measured above the tubers incubated in air, only. When extra ethylene was added to the system, the ethylene concentration was

Table 2

Ethylene production by tubers inoculated with *E. atroseptica*, incubated in air (μl.kg^{-1}.h^{-1})

Incubation time (day)	Experiment I		Experiment II	
	Inoculated	Control	Inoculated	Control
1	—	—	0.05	0.04
2	—	—	—	—
3	—	—	0.09	0.03
4	—	—	0.09	0.06
5	—	—	0.13	0.07
6	—	—	0.15	0.08
7	—	—	0.13	0.06
8	0.25	0.08	0.15	0.07
9	0.32	0.12	0.17	0.07
10	0.31	0.11	0.23	0.08
11	—	—	—	—
12	—	—	—	—
13	0.20	0.10	0.22	0.09
14	0.21	0.08	0.30	0.13
15	0.19	0.06	0.41	0.20
16	0.12	0.06	0.46	0.21
mean	0.23	0.09	0.20	0.09
st. dev.	0.065	0.022	0.119	0.054
t-test	4.548***		3.450***	

Exp. I tubers stored for 1 year at 3°C
Exp. II freshly harvested tubers
— no data

Significance: *P = 5%
**P = 1%
***P = 0.1%

not measured, the method not being sensitive enough for detecting such small differences. In both experiments the Erwinia-inoculated tubers evolved more ethylene (significant at P = 0.1%) than the control (wounded and sterile water treated) ones (Table 2).

Effect of ethylene on resistance of tubers to E. atroseptica. The resistance is expressed as the amount of rotted tissue per tuber (g/tuber, fresh weight). It is not, of course, the absolute value of resistance, but a datum for comparison. The results in Table 3 show that no significant difference was found in resistance of tubers incubated in air or in ethylene to *E. atroseptica*. In the second experiment when freshly harvested tubers were investigated greater inequality occurred between two replicas of the same treatment than between the treatments, but no explanation was found for the phenomenon. In control tubers rot did not occur, the weight of suberized tissue round the wound was 0.10—0.30 g/tuber.

Effect of ethylene on phytoalexin production of tubers. The data given in Table 4 show that the ethylene increased slightly the rishitin and lubimin production but significantly suppressed the formation of phytuberin in tubers inocu-

Table 3

Effect of ethylene on resistance of tubers to *E. atroseptica* (weight of rot g/tuber)

Tubers No.	Treat.	Experiment I				Experiment II			
		air		+10 ppm ethylene		air		+14–16 ppm ethylene	
	Repl.	1	2	1	2	1	2	1	2
1		1.22	1.64	2.43	2.35	2.89	7.72	2.38	5.39
2		2.16	1.72	1.31	4.60	3.23	7.70	3.84	1.62
3		1.12	2.06	0.95	1.43	1.92	5.60	4.10	4.26
4		2.60	1.61	0.96	1.57	5.73	1.48	1.92	7.16
5		2.24	2.71	5.37	1.73	5.36	7.50	3.26	7.11
6		6.27	2.61	1.30	1.84	4.11	5.05	1.12	5.24
7		2.22	2.78	0.91	2.23	0.73	6.96	1.37	2.18
8		3.07	2.44	2.87	2.36	3.29	1.06	1.16	5.10
9		3.25	1.81	1.45	4.10	5.54	6.55	2.03	1.60
10		2.32	2.62	5.47	1.31	5.58	6.33	1.84	6.93
11				2.54	0.88				
mean		2.65	2.20	2.32	2.26	3.84	5.59	2.66	4.66
st. dev.		1.37	0.45	1.60	1.11	1.64	2.32	1.05	2.08
t-test		0.987		0.102		1.948*		2.715*	
mean		2.42		2.29		4.72		3.46	
st. dev.		1.044		1.379		2.191		2.037	
t-test		0.343				1.886			

Exp. I tubers stored for 1 year at 3°C
Exp. II freshly harvested tubers

Significance: *P = 5%
**P = 1%
***P = 0.1%

Table 4

Effect of ethylene on phytoalexin production of tubers inoculated with *E. atroseptica*

Exp.	Treatment	Mean wt of rot g/tuber	PAs in rotted tissue μ/g fr. wt		
			Rishitin	Phytuberin	Lubimin
I	air	2.42	399	1214	8
	+10 ppm ethylene	2.29	493	402	12
II	air	4.72	282	448	4
	+14–16 ppm ethylene	3.46	304	188	6

PAs = phytoalexins
The data are means of two separate replicas
Exp. I tubers stored for 1 year at 3°C
Exp. II freshly harvested tubers

lated with *E. atroseptica*. Approximately two times more phytuberin was detected in the Erwinia-treated tubers incubated in air than in those incubated in presence of 14–16 ppm ethylene.

No phytoalexin was found in tubers treated with sterile water and exposed to ethylene.

Discussion

In both experiments the respiration of inoculated tubers increased compared to the control ones (Table 1). The difference in CO_2 production was greater (significant at $P = 0.1\%$) when the tubers were incubated in air than in presence of ethylene (significant at $P = 5\%$). It is well known that damage or infection increases the respiration of plants, and that exposure to ethylene also promotes the respiration (BURTON, 1952; REID and PRATT, 1972). PRATT and GOESCHL (1960) showed that plant tissues produced abundant ethylene when damaged. In this respect our results were in close agreement with their findings (Table 2). REID and PRATT (1972) postulated that the increased respiration of potato tubers subsequent to damage may be induced by the endogenous ethylene produced by the damaged tissues. Considering STAHMANN's data 1966) that the ethylene induced resistance in sweet potatoes either when the root was damaged or when intact roots were treated, REID and PRATT (1972) also suggested that ethylene could be a "wound hormone" evolved by the damaged tissue as an initial response to wound, and this endogenous ethylene induced the wound respiration and associated processes of repair. In contrary, our results show, that 14–16 ppm ethylene, externally added to the atmosphere where inoculated tubers were incubated, supressed the respiration (expressed in CO_2 production) of Erwinia-infected tubers significantly ($P = 1\%$). The respiration of control (wounded and sterile water treated) tubers did not change significantly if the incubation took place in the presence of ethylene. Furthermore, according to data presented in Table 3, however, less rots were produced in tubers incubated in presence of ethylene, the resistance to *E. atroseptica* did not change significantly as the result of ethylene treatment.

Little increase was noticed in the rishitin and lubimin production of inoculated tubers exposed to ethylene, but it does not seem significant (Table 4). The role of rishitin in resistance is debated. KIRÁLY *et al.* (1972) and BARNA *et al.* (1972) stated that the phytoalexins were not the cause but the consequence of resistence of plants to fungal disease. LYON and co-workers (1975) did not find correlation between the resistance and phytoalexin production of tubers of eight potato cultivars, however they emphasized that rishitin formation was in connexion with resistance and it played an important role in resistance to *E. atroseptica*. No close correlation was found between resistance to *E. atroseptica* and rishitin production in three potato cultivars during storage, and it was suggested that the role of rishitin in resistance could not be a primerily important one (BECZNER *et al.*, 1975).

Considering the LD-value of rishitin for *E. atroseptica* (360 μg/ml in vitro, LYON and BAYLISS, 1975) the little change noticed in its concentration in the ethylene treated tubers, we do not think it would influence the resistance even if the rishitin played a primary role in the inhibition of spread of infection.

The phytuberin production of infected tubers was suppressed by ethylene treatment. The phytuberin has no effect on *E. atroseptica* in vitro up to the concentration of 800 μg/ml (LYON and BAYLISS, 1975). Its suppression by ethylene treatment suggests that phytuberin formation is entirely independent that of the rishitin and very likely does not take part in the resistance of tubers to *E. atroseptica*.

Though the lubimin is active against *E. atroseptica* (BECZNER and LUND 1975), it is likely that the concentration of it required to significantly inhibit this bacterium is higher than that found in infected tissue.

No phytoalexin production was estimated in control, sterile water treated tubers exposed to ethylene. It means, that in contrary to pea and carrot, in potato tubers the phytoalexin formation cannot be induced by ethylene treatment only. In addition, it seems that an exposure of healthy tubers to ethylene will not increase the resistance of tubers to Erwinia, since it did not influence the resistance of infected tubers, in contrast with sweet potato (STAHMANN *et al.*, 1966).

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Virus Diseases of *Solanum dulcamara* L. in Hungary

I. Dulcamara mottle virus

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A virus was isolated from naturally infected Solanum dulcamara L. plants showing symptoms of mild mosaic and slight puckering of the leaves. According to the symptoms on herbaceous plants, physical properties in vitro, properties of purified preparations, serology and electron microscopical evidence this virus was identified as a Hungarian strain of dulcamara mottle virus (DMV—H). This apparently the first report about the presence of dulcamara mottle virus (DMV, R/1 : X/37 : S/S : : S/C1) out of England.

Some new experimental host plants in *Solanaceae* are *Capsicum annuum*, *Datura innoxia*, *D. quercifolia*, *Hyosciamus niger*, *Nicandra physaloides*, *Nicotiana megalosiphon*, *N. tabacum* cv. Samsun (local), *N. rustica*, *Solanum nigrum spp. S. eu-nigrum*, *S. rostratum*. The DMV—H infects *Ammi majus*, *Antirrhinum majus*, *Tetragonia tetragonoides* only locally and *Ocimum basilicum* locally and systemically too. These hosts belong to the families *Aizoaceae*, *Labiatae*, *Scrophulariaceae* and *Umbelliferae* in which were never shown before plants to be susceptible to DMV.

According to the homologous and heterologous titers it was evident that the DMV—H was closely related to DMV and belladonna mottle virus (BMV—H, R/1 : 2.0/37 : S/S : S/C1), and distantly related to eggplant mosaic virus (EMV, R/1 : 2.5/36 : S/S : S/C1). The DMV—H did not give cross reactions with Andean potato latent virus (APLV) and turnip yellow mosaic virus (TYMV, R/1 : 1.9/34 : : S/S : S/C1). Spur formations in agar gel double diffusion test showed that DMV—H was the most closely related to DMV, but it was not identical with it. It can be considered as a serotype of DMV.

Solanum dulcamara L. is a woody drug plant propagated vegetatively. A certain line of *S. dulcamara*, which has a high amount of alcaloids important in the pharmaceutical industry, was seriously damaged by virus infection. The virus infection made almost impossible the propagation of this *S. dulcamara* line, because the virus infected plants grew very slowly, remained stunt. The Research Institute for Medicinal Plants sent several plant material to our Institute for virus identification. Sap inoculation from a number of *S. dulcamara* to herbaceous plants revealed that these plants contained two viruses which were easily separated on *Nicotiana glutinosa* L. and *N. tabacum* L. One of them infected systemically *N. glutinosa* and did not infect *N. tabacum*, the other infected systemically *N. tabacum* and produced only local lesion on inoculated *N. glutinosa* leaves. The host range tests and the electron microscopical investigation of the virus that infects

systemically *N. glutinosa*, proved that the virus should be very closely related or identical to dulcamara mottle virus (R/1 : x/37 : S/S : S/C1) described by GIBBS *et al.* (1966). The present paper reports the identification of this virus in Hungary.

Materials and Methods

The virus (DMV—H) was isolated from *S. dulcamara* sent by Research Institute for Medicinal Plants, Budapest, and maintained in N. glutinosa. The naturally infected *S. dulcamara* plants were replanted in clay pots and placed in an ordinary greenhouse.

Isolates of tymoviruses were kindly provided by Drs. R. KOENIG (dulcamara mottle virus (DMV), eggplant mosaic virus (EMV, R/1 : 2.5/36 : S/S : S/C1)), J. HORVÁTH (belladonna mottle virus (BMV, R/1 : 2.0/37 : S/S : S/C1), turnip yellow mosaic virus (TYMV, R/1 : 1.9/34 : S/S : S/C1)).

Transmission experiments in an insect proof greenhouse took place by mechanical inoculation. Aphids were controlled by chemical treatments at two weakly intervals. The plants were inoculated mechanically when they had developed two leaves or more in steam sterilized soil. Inocula were prepared by grinding infected leaves of *N. glutinosa*, 10 to 15 days after infection together with 0.067 *M* Na-phosphate buffer, pH 7.0 in sterile mortars. Inoculation were carried out with glass spatulas. Carborundum was used as an abrasive. Inoculated plants were rinsed with tap water immediately after rubbing. In each experiment 6 plants of one species were used. After inoculation the test plants were examined for symptoms during one month. Then reisolations of DMV—H were attempted with sap from the rubbed and from the top leaves of each species separately. Assay plant was *N. glutinosa*.

The source of DMV—H for thermal inactivation and aging in-vitro experiments was sap-extracted from *N. glutinosa* inoculated 2—3 weeks earlier. The different treatments were assayed on *N. glutinosa*. Treatment were carried out according to the methods suggested by BOS *et al.* (1960).

The DMV—H infected *N. glutinosa* leaves were harvested 14 to 17 days after inoculation. The leaves were frozen at —15°C. Preparations of DMV—H were purified by the method of GIBBS *et al.* (1966) with slight modification. DMV—H was purified by extracting infected leaves in 0.06 *M* Na-phosphate buffer, pH 6.98, containing 0.1 % thioglycollic acid and 0.05 *M* ascorbic acid and in addition chloroform and n-butanol. After three differential centrifugation cycles (6000 *g*, 10 min, 100 000 *g*, 180 min) we further purified the virus with a linear sucrose-gradient (10—40 %) centrifugation at 24 000 *rpm* (rotor SW 27) for 120 min on Beckman L3—50 ultracentrifuge. The purified preparation was stored in 0.03 *M* Na-phosphate buffer, pH 6.98 at 4° C. The UV absorption spectra were determined by Specord UV VIS spectrophotometer. For immunisation and serological comparison DMV, BMV—H, EMV were purified by the described method.

Isolation of viral RNA for UV-spectrum analyses was made by the phenol-procedure (GIERER and SCHRAMM, 1956). RNA concentrations were determined from their absorbance at 260 nm using $E^{0.1\%}_{1\,cm,\,260\,nm} = 25.0$.

The dry weight determination was repeated five times with 0.2 ml highly purified virus. The dry weight in mg/ml and the extinction value at 260 nm of the same sample were used to calculate the specific extinction coefficient of the DMV–H.

Electron microscopical investigations were carried out on OPTON EM 9 S–2. Samples in 0.03*M* Na-phosphate buffer were placed on carbon-formvar-coated grids and stained with 2% phosphotungstic acid adjusted to pH 6.5 with KOH.

For antiserum to DMV–H, DMV and BMV–H, we immunized a rabbit one time with about 10 mg virus in 2 ml 0.03 *M* Na-phosphate buffer emulsified with 2 ml Freund's complete adjuvant. Bleedings began 14 days after injection and repeated 7–10 days intervals.

The serological investigations were performed by the method of Ouchterlony-double immundiffusion test. The agar-gel consisted of 0.8% Difco Agar Noble, and 0.02% NaN_3 in physiological saline solution. Dilution series of antisera and antigens in crude sap or purified virus were prepared with saline containing 0.05% NaN_3.

Results

Symptoms on source plants and isolation of the virus

In sap inoculation experiments carried out in 1974 and 1975 all plants of *S. dulcamara* exhibited symptoms of mild mosaic and slight puckering of the leaves (Fig. 1).

In this respect the symptoms were very similar to the description reported by GIBBS *et al.* (1966). Infected plants were stunt, the leaves were smaller than that of the healthy plants under the same condition.

Transmission experiments were repeated many times during the two years and the viruses isolated from *S. dulcamara* plants maintained in our greenhouse caused the same severe systemic infection on *N. glutinosa.*

Symptoms on herbaceous hosts

The results of host range tests are given in Table 1. The Hungarian isolate of dulcamara mottle virus did not infect *Chenopodium amaranthicolor* Coste et Reyn., *C. murale* L., *C. quinoa* Willd., *Cucumis sativus* L., *Nicotiana langsdorffi* Weinm., *Phaseolus aureus* Roxb., *P. vulgaris* L. cv. Pinto, *Pisum sativum* L. cv. Rajnai törpe, *Vigna sinensis* L., *Vicia faba* L.

Stability of DMV–H in vitro

Sap extracted from *N. glutinosa* and assayed on *N. glutinosa* remained infectious when heated to 80°C for 10 minutes. However, heating to 85°C resulted

in no infection. It should be emphasised that most of the infectivity was lost after heating for 10 min at 70–75°C and only one or two of 12 *N. glutinosa* showed symptoms after 10 min heating at 78°C or at 80°C but not at higher temperature. Sap containing DMV–H usually remaind infective at laboratory temperature (about 24°C) for only 5–7 days and at 4°C for more than 2 months. Appearance of symptoms was very strongly influenced by post-inoculation temperature. The symptoms were slower to develop at 15°C or at 28°C or 30°C than at 20–22°C.

Properties of purified preparation

Extracts of plants of *N. glutinosa* infected with DMV–H contained a number of approximately spherical particles. They were never found in preparation

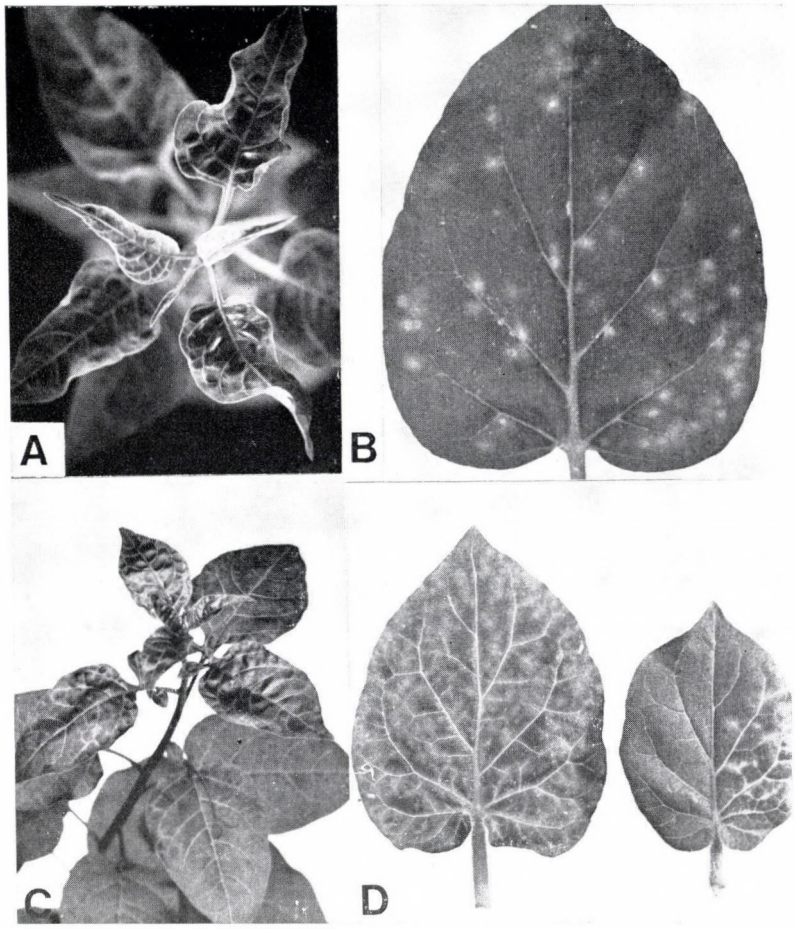

Fig. 1. Symptoms on the naturally infected (A) and the experimentally inoculated (B) *Solanum dulcamara* with DMV–H; Local lesion (C) and systemic symptoms (D) on the experimentally inoculated *Nicotiana glutinosa*

of healthy plants. The described purification method yealded reasonable amounts of virus. Purified preparations, centrifuged on sucrose density gradient columns for two hours with rotor of SW27 at 24 000 rev/min formed two light-scattering zones (Fig. 2). The top component contained empty particles while the bottom consisted of the intact virus (Fig. 2E, F). Both were serologically active.

The UV-absorption spectrum of highly purified virus with a maximum at 260–262 nm and a minimum at 242–244 nm was typical of a nucleoprotein which contained a high amount of nucleic acid. The E_{260}/E_{280} was 1.72–1.78 and E_{280}/E_{260} was 0.60–0.72. The E_{max}/E_{min} was 1.39–1.44. The slight differences within the extinction ratios obtained pointed out there are differences between the amounts of empty and full particles in different purified preparation. The DMV–H–RNA

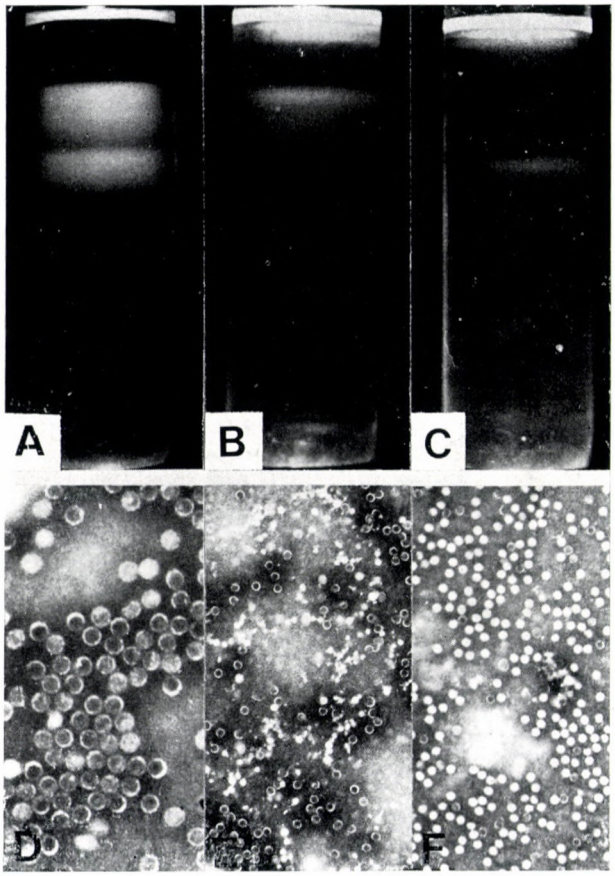

Fig. 2. Results of centrifuging in a sucrose gradient (10–40%) in a SW 27 rotor at 24 000 rev./min. for 2 h. A = unfractionated DMV–H preparation B = separated top component, C = separated bottom component. Electron micrographs of a unfractionated preparation of DMV–H (D), of a top component (E), and of a bottom component (F)

Table 1

Host range and symptomatology of Hungarian isolate of dulcamara mottle virus

Family and binominals	Reactions	
	Inoculated leaves	Uninoculated tip leaves
Solanaceae		
Capsicum annuum L.	symptomless infection	green mosaic, mild mottle
Datura innoxia Mill.	chlorotic or necrotic lesions	necrosis, chlorotic and necrotic spots, mosaic with yellow islands
Datura quercifolia H. B. et K.	necrotic lesions	mosaic with chlorotic or necrotic spots
Datura stramonium L. H. B. et K.	chlorotic or necrotic lesions	chlorotic and necrotic spots, mild mosaic
Hyosciamus niger L.	chlorotic lesions	not infected
Lycopersicon esculentum Mill.	symptomless	mosaic with yellow islands, malformation
Nicandra physaloides (L.) Gaertn.	chlorotic lesions	veinclearing, necrotic spots, malformation
Nicotiana clevelandii Gray.	chlorotic or necrotic lesions	veinclearing, green mosaic, necrotic spots, stunting
Nicotiana glutinosa L.	chlorotic local lesions with necrotic center	veinclearing, chlorotic and necrotic spots, mosaic with yellow islands, stunting
Nicotiana megalosiphon Heurck et. Meull.	chlorotic or nectotic lesions	veinclearing, necrotic flecks, mosaic, stunting
Nicotiana tabacum L.		
cv. Bel 61—10	not infected	not infected
cv. Samsun	symptomless infection occasionally	not infected
cv. Xanthi-nc	not infected	not infected
Nicotiana rustica L.	chlorotic or necrotic lesions	veinclearing, mosaic
Petunia hybrida Vilm.	small necrotic lesions	symptomless infection or mild mosaic
Solanum dulcamara L.	chlorotic lesions	mild mottle, stunting
Solanum dulcamara L. var. flore-alba	chlorotic lesions	mild mottle, stunting
Solanum nigrum L.	chlorotic or necrotic lesions	veinclearing, yellow-green mosaic
Solanum nigrum L. spp. S. eu-nigrum	chlorotic or necrotic lesions	veinclearing, yellow-green mosaic
Solanum rostratum Dun.	brown-red local lesions	mosaic with necrotic spots
Aizoaceae		
Tetragonia tetragonoides (Pall.) O. Ktze.	chlorotic lesions	not infected
Labiatae		
Ocimum basilicum L.	small necrotic lesions	mild mottling
Scrophulariaceae		
Antirrhinum majus L.	chlorotic lesions	not infected
Umbelliferae		
Ammi majus L.	symptomless infection	not infected

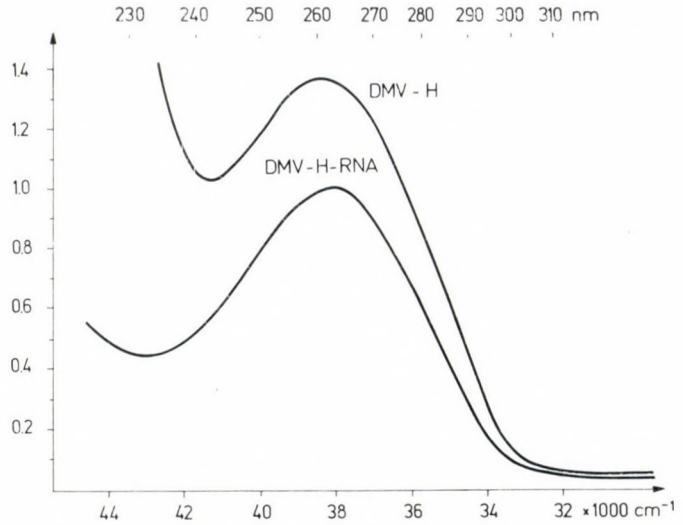

Fig. 3. The U.V. absorption of the Hungarian strain of dulcamara mottle virus and its nucleic acid. The concentrations of DMV—H and its RNA were 0.17 mg/ml and 0.04 mg/ml, respectively

had a maximum and a minimum absorption at 263 and 234, respectively, with E_{260}/E_{280} and E_{max}/E_{min} absorption ratios of 1.72 and 2.24 (Fig. 3). The specific extinction coefficient of the virus was $E^{0,1\%}_{1\,cm,\ 260\,nm} = 8.1 \pm 0.1$.

Serology

Many of the properties of DMV—H closely resembled not only to those of dulcamara mottle virus but also of tymoviruses.

Crude sap and purified preparations of DMV—H were therefore tested in gel double diffusion tests against its homologous antisera and against antisera of several tymoviruses. The results are presented in Table 2.

According to the homologous and heterologous reactivity it is evident that the DMV—H is closely related to DMV and BMV—H. DMV—H, DMV and BMV—H are so closely related that their distinction is impossible with their only homologous antisera. In this respect our results agreed completely with the conclusion of KOENIG and GIVORD (1974). The DMV—H is distantly related to EMV however serological relationship with Andean potato latent virus (APLV) or TYMV was not detected.

When DMV—H or BMV—H and DMV—G antisera were in the central well is separate experiments and homologous and heterologous viruses were placed in surrounding wells, spur were formed, indicating heterologous reactions among DMV, DMV—H, BMV—H and EMV. Figs4 and 5 show the homologous and heterologous reactions in agar gel-diffusion tests. According to the homol-

Table 2

Homologous and heterologous titers of antisera to Hungarian isolate of dulcamara mottle virus and some members of tymovirus group

Antiserum to	Antiserum sample[1]	Antigen									
		DMV-H		DMV[2]		BMV-H[3]		EMV[2]		TYMV[3]	
		crude sap	purified	crude sap	purified	crude sap	purified	crude sap	purified	crude sap	purified
DMV-H[5]	14	512	512	—	—	64	256	—	—	—	—
	16	256	256	256	128	64	64	16	16	0	—
	36	1024	1024	1024	512	256	256	64	64	0	—
	42	1024	1024	512	1024	512	512	128	128	0	—
DMV-B[2]		512	256	1024	1024	512	256	32	32	0	—
DMV-G[5]	21	512	256	1024	1024	512	256	4	4	0	—
BMV-H[5]	14	64	64	64	64	512	512	8	8	0	—
	19	256	256	512	512	1024	1024	0	0	0	—
	32	512	512	1024	512	2048	1024	8	8	0	—
	43	512	512	1024	512	2048	1024	8	8	0	—
	51	1024	1024	1024	512	4096	4096	8	8	0	—
	72	512	512	1024	512	4096	4096	16	16	0	—
EMV[2]		16	32	16	16	8	8	512	512	0	—
APLV[2] (1024)		0	0	0	0	0	0	16	32	0	—
TYMV-Scott.[4]		0	0	0	0	0	0	0	0	512	—

1 = Number of days after the first injection; 2 = EMV, DMV and antisera of DMV-B, EMV, APLV; 3 = BMV-H and TYMV; 4 = antiserum of TYMV kindly provided by R. Koenig, J. Horváth and N. Juretic respectively; 5 = DMV-H, DMV-G and BMV-H antisera were prepared in our laboratory

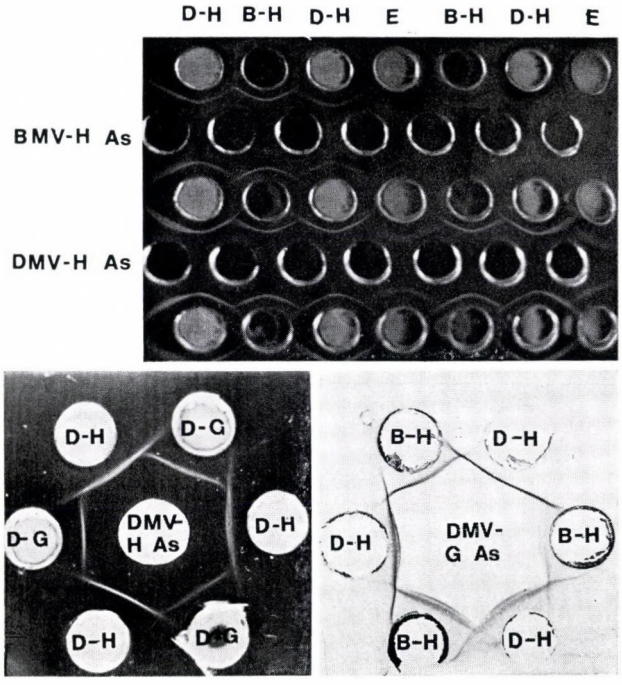

Fig. 4. Homologous and heterologous reactions among the DMV—H, DMV, BMV—H and EMV. Abbreviations of antigens: D—H = DMV—H, D—G = DMV, B—H = = BMV—H, E = EMV

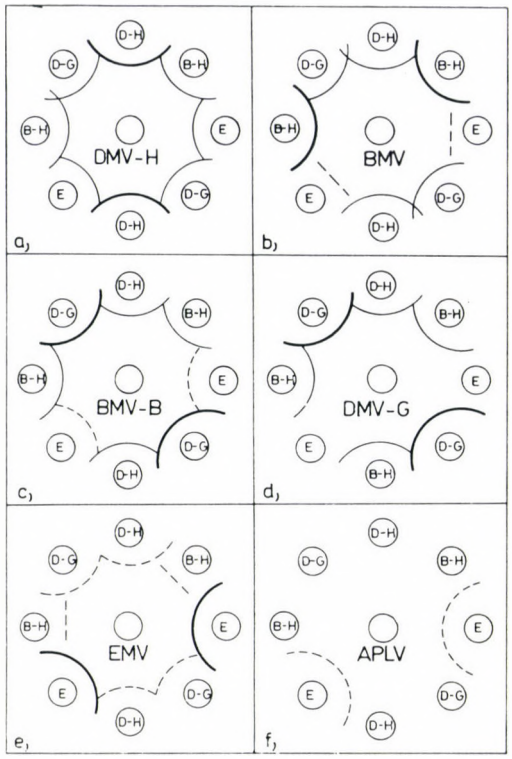

Fig. 5. Immundiffusion tests with DMV—H, DMV—G, BMV—H and EMV antigens. A. Central well is filled with antiserum DMV—H and surrounding wells with homologous DMV—H or heterologous antigens. Heterologous reactions appear with DMV—G, BMV—H and EMV. DMV—H antiserum reacts with BMV—H and DMV—G resulting precipitin bands crossing each other in the pattern of non-identity; B. Central well is filled with antiserum BMV—H and surrounding wells with homologous BMV—H and heterologous antigens. Heterologous reactions appear with DMV—H and DMV—G and a stright diffusion zone with EMV. The precipitin bands of DMV—H and DMV—G cross each other indicating that BMV-antiserum posesses antibodies which are specific to DMV—H or only DMV—G; C. Central well contains antiserum DMV—B; Heterologous reactions appear with DMV—H, BMV—H and EMV. Cross spurs were with DMV—H and BMV—H; D. Central well contains antiserum DMV—G; Heterologous reactions appear with DMV—H, BMV—H but no reaction with EMV. Precipitin band of BMV—H does not cross the precipitin band of DMV—H indicating that this antiserum does not possess antibodies which are specific to BMV only, but not to DMV—H; E. Central well is filled with antiserum EMV; Heterologous reactions appear with DMV—H and DMV—G. The BMV—H reacts only with a very fine diffusion zone; F. Central well contains antiserum APLV. Faint reaction is only seen with EMV

ogous and heterologous reactions it was evident that DMV—H was not identical with DMV, BMV—H and EMV. The homologous and heterologous reactions of DMV—H, DMV and BMV—H showed that each antiserum contained anti-

bodies which reacted with the other viruses, too, and antibodies which reacted with one of the other two only. The amount of the latter type of antibodies was so small, that the cross-spurs appeared in case of optimal antigen–antibody concentration only.

The DMV–B and DMV–G antisera were prepared against the same isolate of dulcamara mottle virus in Braunschweig (DMV–B) and in Budapest (DMV–G). DMV–G antiserum does not possess antibodies which led to cross-spurs formation between DMV–H and BMV–H. The precipitin band of DMV–H produced contained more specific antibodies to DMV–H than to BMV–H.

All these results led us to the conclusion that the DMV–H was in closer serological relationship with DMV than with BMV–H. They also indicated that the two DMV isolates were in more distant relationship with EMV than with BMV–H.

Discussion

According to some reviews *S. dulcamara* can be susceptible to the following viruses: Andean potato latent virus, beet pseudo-yellows virus, citrus exocortis virus, potato aucuba mosaic virus, potato leaf roll virus, potato virus X, potato virus Y, tobacco mosaic virus, tobacco necrosis virus, tobacco rattle virus, tobacco streak virus and tomato spotted wilt virus (cf. THORNBERRY, 1966; cf. KLINKOWSKI, 1968; cf. SCHMELZER and WOLF, 1971; cf. SMITH, 1972).

Some of the above mentioned viruses infect *S. dulcamara* only under experimental condition. GIBBS *et al.* (1966) reported a new virus disease of *S. dulcamara* caused by dulcamara mottle virus. As far as we know this virus was found in England only. This virus belongs to the tymovirus group. *S. dulcamara* can be an important natural host of cucumber mosaic virus (GIBBS *et al.*, 1966; DEVERGNE, 1976) and plum pox virus (KRÖLL, 1971), too. In Hungary GÁBORJÁNYI and NAGY (1972) isolated the alfalfa mosaic virus from *S. dulcamara.*

The characteristic local and systemic symptoms on *N. glutinosa* caused by our virus isolate from *S. dulcamara*, its narrow host range mostly limited to *Solanaceae* and electron microscopical investigations proved that this virus is similar or probably identical with dulcamara mottle virus described by GIBBS *et al.* (1966). It is unlikely that confusion can be made with hitherto described viruses attacking *S. dulcamara* when we consider the results of symptomatology, serology and electron microscopical evidence.

The host range for the Hungarian isolate of DMV is in agreement with the findings of GIBBS *et al.* (1966). Some discrepancy seems to occur concerning the infection of DMV–H on *Petunia hybrida* which can be explained by different source of seeds of Petunia, different experimental conditions and also differences between strains of DMV. Some new artificial host plants in *Solanaceae* are *Capsicum annuum*, *Datura inoxia*, *D. quercifolia*, *Hyosciamus niger*, *Nicandra physaloides*, *Nicotiana megalosiphon*, *N. tabacum* cv. Samsun (local), *N. rustica*, *Solanum*

nigrum spp. S. eu-nigrum, S. rostratum. The DMV–H infects *Ammi majus, Antirrhinum majus, Ocimum basilicum* and *Tetragonia tetragonoides.* The new host plants belong to the families *Aizoaceae, Labiatae, Scrophulariaceae* and *Umbelliferae* in which were never shown before plants to be susceptible to DMV. The wider host range of DMV–H than that of DMV is only an apparent difference between the two viruses because these new hosts were not investigated in earlier experiments with DMV.

DMV–H shares most of its experimental hosts among solanaceous species with APLV, EMV, BMV and some recently described viruses, *Physalis* mosaic virus (PMV, PETERS and DERKS, 1974) and *Physalis mottle* strain of belladonna mottle virus (PM–BMV, MOLINE and FRIES, 1974). In spite of that DMV–H can be distinguished on some differential host plants listed in Table 3.

The shape and size of the particles, the behaiour of the virus in sugar density centrifugation, the way of transmission and properties in vitro of the Hungarian DMV-isolate are in close agreement with data found in the literature for DMV.

Spur formations in agar gel double diffusion test shows that DMV–H is most closely related to DMV, but it is not identical with it. It can be considered as a serotype of DMV.

The homologous and heterologous reactivity of DMV–H, DMV and BMV proved the statement that in case of viruses within morphological groups there is a continuum of serological relationships which was determined in tymovirus-group (KOENIG and GIVORD, 1974) and potyvirus-group (BECZNER *et al.*, 1976). According to KOENIG (1976), BMV and DMV form a closely interrelated group inside of tymoviruses and might possible be regarded as a strains of the same virus. Our opinion based on host range differences is they should be differentiated as separate viruses.

In serological tests, the DMV–H did not give cross reactions with APLV and TYMV. Based on heterologous reactions among DMV–H, DMV, BMV and EMV, we have placed DMV–H in the Andean potato latent subgroup of the turnip yellow mosaic virus group. In our experiments the heterologous reactions

Table 3

Differential host range for Hungarian isolate of dulcamara mottle virus and some related viruses

Host	DMV-H	DMV	APLV	EMV	BMV	PMV	PM-BMV
Nicotiana glutinosa L.	LS	LS	LS	LS	LS	L	LS
N. tabacum L. cv. Xanthi	–	–	?	L(S)	LS	L	?
Chenopodium quinoa L.	–	–	LS	LS	–	–	L

L = local infection, S = systemic infection, ( ) = infection occasionally, – = no infection, ? = no information. The type of reactions presented are compiled from findings of Gibbs et al. (1966), Paul et al. (1968), Gibbs and Harrison (1969), Moline and Fries (1974), Peters and Derks (1974) and the present authors

of EMV with APLV-antiserum was the indirect evidence that the DMV–H belongs to the above mentioned subgroup. According to such kind of serological relationships with scrophularia mottle virus and among the member of APLV subgroup, and TYMV subgroup KOENIG and GIVORD (1974) demonstrated a continuous range of serological relationship within the tymovirus group and concluded that a subdivision into a turnip yellow mosaic and an Andean potato latent virus subgroup is not justified.

Dealing with the bean yellow mosaic virus and the more or less related viruses BOS (1970), BOS *et al.* (1974) and BECZNER *et al.* (1976) concluded that the identification of an intermediate isolate having overlapping characters with related viruses the borderlines had to be drawn arbitrarily and they would not be sharp-cut between the artificially defined taxonomic entities.

In case of identification of DMV–H, the physical properties in vitro, the properties of purified virus and the serology are important criteria to place this virus in tymovirus-group because of many overlapping characters. These properties give little help to differentiating the DMV–H and related viruses. But based on host range or rather on pathological behaviour of DMV–H we could conclude that this virus is a new strain of DMV, we have provisionally named in Hungarian strain of dulcamara mottle virus.

The discovery of the DMV–H in Hungary is important from several standpoints. It provides evidence that DMV can have a wide geographical distribution outside England or if it was not an indigenous virus in Hungary it was introduced by vegetative plant material. Furthermore, beside of the BMV (HORVÁTH *et al.*, (1976) and TYMV (JURETIĆ *et al.*, 1973) the Hungarian strain of dulcamara mottle virus was the third member of tymovirus group described in Hungary.

Acknowledgements

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Inhibition of Cucumber Mosaic Virus by Some Chemicals

By

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Surfactants, dyes, base analogues and antibiotics were tested for their inhibitory action against a strain of cucumber mosaic virus isolated from bottlegourd. Crystal violet, sodium lauryl sulphate and thio-uracil completely inhibited the virus at concentrations between 100 and 1000 ppm when mixed with the inoculum. Inhibitory action was better at neutral or acidic pH. The virus was inactivated completely in leaf discs floated in solutions containing different chemicals. Increase in time of contact between inhibitory substances and the virus *in vitro* increased the rate of inhibition. Treatment of systemic hosts with sodium lauryl sulphate and thiouracil delayed appearance of symptoms. Plants sprayed with various chemicals contained less virus than the controls. The inhibitory action of chemicals was more pronounced in local lesion hosts. Calli of diseased tissues grown in growth medium containing 10 ppm thiouracil contained less virus than in calli grown in ordinary medium.

Cucumber mosaic virus and its numerous strains affect cultivation of a large number of crops and ornamentals. As these viruses are transmitted by aphids, mainly efforts were directed for devising methods to check the vectors of these viruses, but as many of these viruses are also carried through seeds or parts used for vegetative propagation, cure of affected plants or parts thereof is imperative. A wide variety of chemicals have been tested for inhibition of different plant viruses with varying success (Bawden, 1954; Matthews and Smith, 1955; Raychaudhuri, 1966). We tested surfactants, dyes, base analogue and antibiotics for inhibition of cucumber mosaic virus isolated from bottlegourd and the results are reported here.

Materials and Methods

Virus

Cucumber mosaic virus, isolated from bottlegourd *(Lagenaria siceraria)* was maintained in bottlegourd cv. Prolific Long and also *N. tabacum* cv. White Burley.

Assay hosts

Lagenaria siceraria was used for systemic infection and *Chenopodium amaranticolor* for local lesion assay.

Transmission

The virus was transmitted by sap inoculation to test plants.

Chemicals tested for virus inhibition

Aflatoxin (Central Food Technology Research Institute, Mysore), Blasticidin S (BS*; Kaken Chemical Co., Ltd., Tokyo), crystal violet (CV*; E. Merck Ag. Darmstadt), 2,4-dichlorophenoxy acetic acid (2,4-D; BDH, London), sodium lauryl sulphate (SLS*; BDH, London) and thiouracil (TU*; C_2-mercapto, 4-hydroxypyrimidine; Nutritional Biochemicals Corporation, Cleveland, Ohio) were tested. Solution of thiouracil was prepared in dilute ammonium hydroxide; 2,4-D was first dissolved in 2 ml of absolute alcohol and then solution prepared in double distilled water; solutions of other chemicals were prepared in double distilled water.

The inoculum was mixed with chemicals ten minutes prior to inoculations. The leaves were thoroughly washed with tap water after inoculations. For systemic host assay, the chemicals were either sprayed with an atomizer on leaves or applied through cotton wick. For assaying concentration ten, 10 mm leaf discs punched from the young apical leaves were macerated in 1 ml of distilled water and extracts assayed on *C. amaranticolor*.

Tissue culture

Calli of tobacco cv. White Burley were grown in MURASHIGE and SKOOG's (1962) medium supplemented with 2,4-D and coconut milk. Calli from diseased plants were subcultured on media containing various inhibitory chemicals; identical pieces of Calli were used for subculturing and the chemicals were added in growth medium before autoclaving. The cultures were maintained at 20 to 24°C and 72 foot candles light, for four weeks. Virus concentration in various calli was assayed on *C. amaranticolor*.

Results

Effect of chemicals on virus infectivity when mixed with the inoculum

All the chemicals inhibited local lesion development in *C. amaranticolor* (Table 1). Maximum inhibition was caused by TU, followed by CV and SLS. At lower concentrations, however, SLS was better than the other chemicals. Aflatoxin and BS also inhibited the virus but were less effective than TU, CV and SLS while 2,4-D was least effective at all the concentrations tested.

BS, CV, SLS and TU were further tested singly and in combination for inhibition of the virus. No additive inhibitory effect were noticed. Maximum inhibition was obtained by BS-SLS followed by BS-CV-TU (Table 2).

* Acronyms used in this paper.

Table 1

Effect of chemicals on the infectivity of the virus

Chemicals tested	Mean percentage inhibition* Concentration in ppm				
	1,000	100	10	1	0.1
Aflatoxin	—	44.71	42.82	41.38	20.44
Sodium lauryl sulphate	90.00	50.83	50.07	42.19	—
Crystal violet	90.00	51.59	46.32	32.90	—
Thiouracil	90.00	90.00	45.63	35.24	—
Blasticidin S	51.00	45.46	38.70	25.18	—
2, 4-D	37.35	22.87	21.64	16.54	—

* = Angular values

	S. Em.	C. D. at 5%	C. D. at 1%
Treatments	2.66 + 1.26	12.90 + 3.49	17.28 + 4.57
Chemicals	1.41	3.90	5.11
Interaction (Chemical × Treatment)	2.82	7.82	10.25

(+ = Values for aflatoxin only as this was tested separately)

Table 2

Effect of chemicals on the infectivity of the virus, in various combinations

Chemicals and their combinations*	Mean percentage inhibition[a]
BS	41.21
CV	45.11
SLS	46.95
TU	46.49
BS + CV	48.04
BS + SLS	52.30
BS + TU	45.80
BS + CV + SLS	49.84
BS + CV + TU	50.65
BS + SLS + TU	47.47
BS + CV + SLS + TU	48.62
CV + SLS	36.93
CV + TU	40.86
CV + SLS + TU	46.38
SLS + TU	39.93

[a] = Angular values

* = Final concentration of each chemical in every combination 10 ppm

	S. Em.	C. D. at 5%	C. D. at 1%
Treatments	2.85	7.90	10.38

Effect of pH on inhibitory action of various chemicals when mixed with the inoculum

The chemicals were prepared in 0.15 *M* phosphate buffer at different pH levels. Inocula in buffers without chemicals were used as corresponding controls. pH 6.2 and 7.0 were better than pH 5.6 and 8.0 for BS and SLS, but CV and TU were more effective at pH 5.6 (Fig. 1). Inhibitory effect of TU depended more conspicuously on the pH of suspending medium.

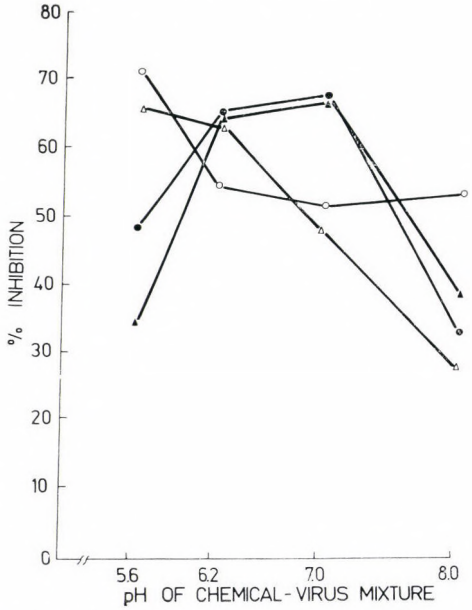


Fig.1. Effect of chemicals on the infectivity of the virus at different pH (●—● SLS; ○—○ CV; ▲—▲ BS; △—△ TU)

Virus concentration in leaf discs floated in media containing inhibitory chemicals

Ten leaf discs of systemically infected bottlegourd leaves were floated in test solutions containing 1000 ppm of inhibitory chemicals and virus concentration in leaf discs assayed at varying intervals. The discs were maintained at 8–10°C. For virus assay discs were washed, dried in folds of filter paper and virus extracted in double distilled water for assay on *C. amaranticolor*. All the four chemicals BS, CV, SLS and TU completely inactivated the virus in leaf discs when floated for 16 hours for SLS and TU and 24 hours for the other two (Fig. 2).

Factors affecting virus inhibition by different chemicals

The percentage inhibition increased by increasing the dilution of virus and maintaining constant concentration of BS, CV, SLS or TU (100 ppm; Fig. 3). A similar effect was found when the virus-chemical-mixtures were also diluted

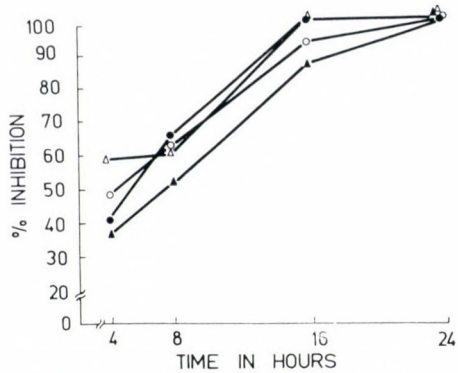


Fig. 2. Concentration of infective virus in leaf discs from d iseased plants floated on solution of different chemicals for various timings (●—● SLS; ○—○ CV; ▲—▲ BS; △—△ TU)

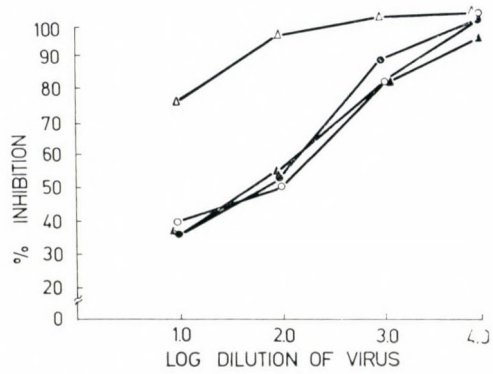


Fig. 3. Effect of addition of constant amount of chemicals to various dilutions of the virus (●—● SLS; ○—○ CV; ▲—▲ BS; △—△ TU)

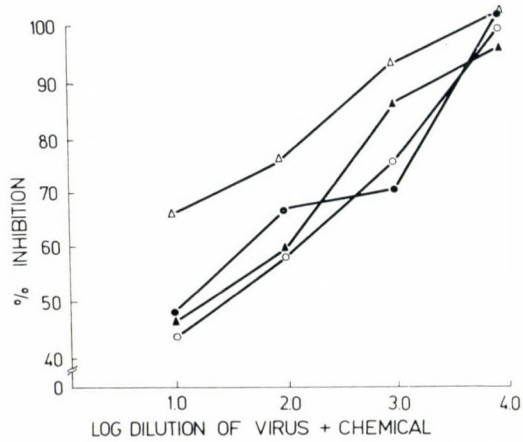


Fig. 4. Effect of dilution of chemical–virus mixtures on infectivity (●—● SLS; ○—○ CV ▲—▲ BS; △—△ TU)

(Fig. 4). Increase in time of contact between the chemicals and the virus by storing mixtures for various timings before inoculation also increased the extent of inhibition by the chemicals. The effect was more pronounced for TU than other chemicals (Fig. 5).

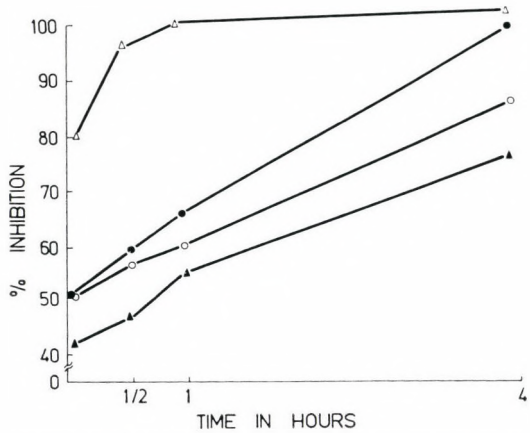


Fig. 5. Effect of period of contact between virus and chemical *in vitro* on virus infectivity (●—● SLS; ○—○ CV; ▲—▲ BS; △—△ TU)

Effect of chemicals when applied to test plants

When chemicals were applied twice i.e. 24 hours and 12 hours before inoculation on abaxial surfaces of leaves of *C. amaranticolor*, and virus inoculated on adaxial surface, all the chemicals inhibited local lesion development (Table 3).

The chemicals (1000 ppm) were similarly applied on the leaf surfaces, of local lesion and systemic hosts, to be inoculated or after inoculation. For local lesion host, chemicals were applied only to one half of leaves, the other half was taken as control. Inhibition with all the chemicals was greater when applied just before or after inoculation (Figs 6 and 7).

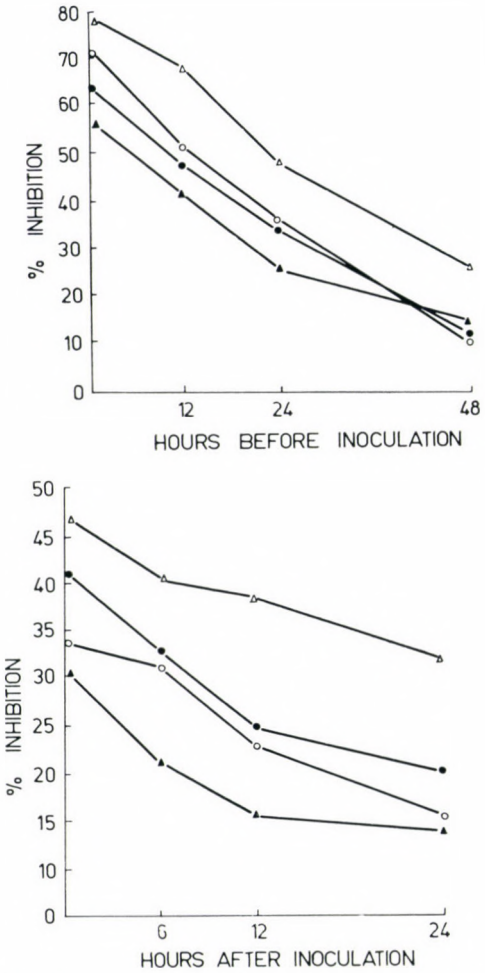
Table 3

Effect of applying chemicals to the lower surface of the leaf

Chemicals tested	Concentration in ppm	Mean percentage inhibition*
SLS	1.000	27.90
Crystal violet	1.000	18.72
Thyouracil	1.000	20.27
Blasticidin S	1.000	21.22

* = Angular values	S. Em	C. D. at 5%	C. D. at 1%
Chemicals	1.42	4.03	5.29

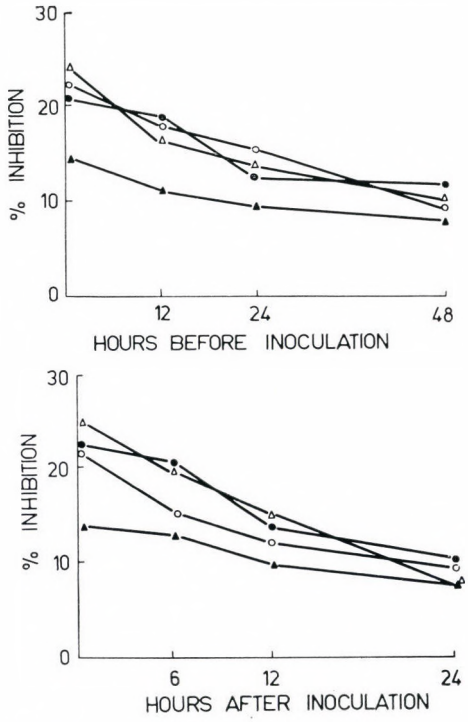
To examine the effect of inhibitory chemicals on the multiplication of the virus various chemicals BS (10 ppm), CV, SLS and TU (100 ppm), were sprayed to the dripping stage with an atomizer on vigorously growing plants of bottlegourd. Twenty plants were used for each treatment. The plants were inoculated at various intervals before and after spraying with chemicals. Concentration of the virus in each plant was assayed on *C. amaranticolor* after four weeks. For bioassay, 10 leaf discs were punched at random and inoculum prepared in 1 ml of distilled water. The concentration of the virus was lower in plants sprayed with chemicals



Figs 6—7. Effect of chemicals on virus infectivity when applied to local lesion host before (Fig. 6) and after (Fig. 7) inoculation (●—● SLS; ○—○ CV; ▲—▲ BS; △—△ TU)

just before or after inoculations (Figs 8 and 9). However, the effect was much less pronounced than in experiments with local lesion host.

Treatment of systemic hosts with these chemicals for longer durations did not add much to advantage. The bottlegourd plants were either sprayed daily or treated with various chemicals through wick method for two weeks starting 24 hours before virus inoculation. The symptoms were obtained daily and virus concentration assayed 4 weeks after inoculation.



Figs 8—9. Effect of chemical on concentration of cucumber mosaic virus in systemic host when sprayed before (Fig. 8) and after (Fig. 9) virus inoculation (●—● SLS; ○—○ CV; ▲—▲ BS; △—△ TU)

The plants treated with BS and CV developed symptoms as early as in control plants and the concentration of virus in these plants was also more than in the other two treatments. Plants treated with SLS and TU developed symptoms 1 to 3 days after the control plants. Maximum delay (3 days) was observed in plants sprayed with TU. SLS and TU inhibited virus multiplication to a similar extent (Fig. 10). The overall reduction in multiplication, although more than with two treatments only, indicates limitation in the extent to which these chemicals can inhibit multiplication.

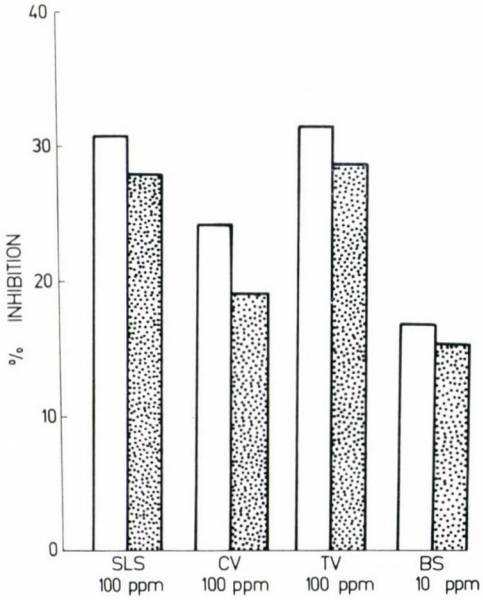


Fig. 10. Effect of chemicals on concentration of the virus in systemic host when applied by spraying (□) or through cotton wick (■)

Effect of chemicals on virus multiplication in tissue culture

SLS (10 and 100 ppm) and TU (10 and 50 ppm) were tested. Calli grew normally in medium containing 10 ppm TU, but in media containing SLS growth was retarded and smaller calli were produced. TU at 50 ppm was toxic. Virus multiplication was inhibited maximum when TU at 10 ppm was incorporated in the culture medium (Table 4). In general the chemicals were more effective when incorporated in growth medium than when sprayed on to diseased plants.

Table 4

Effect of chemicals on the multiplication of the virus in tissue culture

Chemicals tested	Concentration in ppm	Mean percentage inhibition	Angular value
SLS	10	48.68	44.20
SLS	100	52.21	46.26
Thiouracil	10	57.24	49.14
Thiouracil	50	46.41	42.94

	S. Em	C. D. at 5%	C. D. at 1%
For SLS			
Treatments	1.26	N. S.	N. S.
For thiouracil			
Treatments	1.81	5.43	7.52

Discussion

All the chemicals tested inhibited cucumber mosaic virus to varying extents. The inhibition increased with increase in the concentration of chemicals. CV, SLS and TU completely inhibited the virus at concentrations between 100 and 1000 ppm. SLS is also reported to inactivate cowpea banding mosaic virus (CpBMV; SHARMA and VARMA, 1975), a strain of CMV, in similar studies (RAO, 1970). BS was less effective than the other chemicals but, when mixed with other chemicals the efficacy seemed to increase.

The chemicals tested in the present study worked better either at neutral or acidic pH. Unlike sodium dodecyl sulphate which is more effective at alkaline pH (FRANKEL-CONRAT *et al.*, 1957; BAWDEN and PIRIE, 1940), SLS was least effective in alkaline pH.

Dependence of antiviral activity on concentration of the virus in the inoculum, need for spray of chemicals just before or after inoculation for effective inhibition and insignificant activity of the chemicals when applied on the lower surfaces of leaves inoculated with the virus on upper surfaces indicate that the chemicals tested in the present study are more likely to directly affect the virus rather than alter host metabolism. Slight inhibition of the virus when virus inhibitors were applied on lower surfaces and virus inoculated on upper surfaces of the leaves may be due to partial movement of the chemicals in leaf tissues although surfactant DOSS, apparently does not move in leaf tissues (SCHNEIDER and MICHALL, 1962). The possibility of alteration in host metabolism, however, cannot be ruled out. SLS is more likely to act on virus directly as it is known to separate viral protein and RNA (FRANKEL-CONRAT *et al.*, 1957). TU affects virus replication (JEENER, 1965; FRANCKI and MATTHEWS, 1962), therefore, its activity would depend on availability in cells in which virus is replicating. Thus when applied alongwith the inoculum it could inhibit the virus more by preventing initiation of infection. This explains obviously more inhibition in local lesion host than in systemic host as TU is not a total inhibitor.

CV might react with viral nucleic acid and make it non-infectious as with some other dyes (MATTHEWS, 1970). BS probably inhibits virus RNA synthesis in the host (HIRAI *et al.*, 1968).

Although no chemical provided the desired cure or protection of plants from infection with the virus, its complete inactivation in leaf discs floated in solutions containing inhibitors give hope of developing cure for seed borne viruses. Indeed in limited trials soaking of seeds of cowpea carrying CPBMV in TU at 500 ppm for one hour completely freed seeds from the virus which is carried in 15–31% seeds from diseased cowpea (SHARMA and VARMA, 1975).

Acknowledgement

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Reversion of Dwarfing Induced by Virus Infection: Effect of Polyacrylic and Gibberellic Acids

By

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Treatments with polyacrylic acid (1 ml/l Mwt. 230 000) or with gibberellic acid (10 mg/l) in tap water eliminated stunting induced by infection of tobacco etch virus (Cuban isolates: To 42 and To 77) in pepper (cv. California Wonder and Keystone Resistant) and in tomato (cv. Manalucie). Both treatments increased growth of diseased and of healthy plants but did not affect the symptom expression. We suggest that the effect of polyacrylic acid and gibberellic acid is related to cell elongation rather than with antiviral activity.

The most common symptom of systemic virus infection of the plant (compatible host–parasite relationship) is growth inhibition. Both early infection and the severity of the infective strain are influencing the growth rate of the diseased plants. To eliminate this inhibitory effect, the majority of the experiments have used treatments of natural promotive plant hormones against a number of mycoplasmas and viruses (MARAMOROSCH, 1957; HULL and KLOS, 1958; YERKES, 1960; ORLOB and ARNY, 1961; STEIN, 1962; NARIANI, 1963). In one of the earlier works CHESSIN (1957) used gibberellic acid to reverse the stunting effect of tobacco etch virus.

Recently a lot of evidence has been accumulated about the antiviral effect of some polyanions. According to DE SOMMER *et al.* (1968) the virus adsorption is affected, virus multiplication is inhibited and interferon synthesis is promoted by the application of these substances. Similar results were also published by STEIN and LOEBENSTEIN (1972). Treatment with polyacrylic acid, as GIANNINAZZI and KASSANIS (1974) demonstrated induced a *de novo* synthesis of an additional protein, interfering with TMV multiplication. In these experiments, they observed some stimulation in growth of the polyacrylic acid treated plants (GIANNINAZZI, 1975 personal communication). The aim of our recent experiments was to determine how the polyacrylic acid is able to reverse the growth inhibition caused by virus infection. Gibberellic acid was run in parallel experiments because it is a well known material in this effect.

Material and Methods

Green pepper (*Capsicum annuum* L. cv. California Wonder and Keystone Resistant) and tomato (*Lycopersicon esculentum*, L. cv. Manalucie) were grown in ordinary greenhouse conditions and inoculated with two isolates of tobacco etch virus (TEV). The isolates were from naturally infected tomatoes (isolates To 42, To 77, Cf.: FERNANDEZ, 1975), and were increased in tobacco (*Nicotiana tabacum* L. cv. Xanthi-nc.). The centrifuged crude extract of tobacco leaves was diluted two fold with 0.1 *M* Sörensen buffer (pH 7.1) and served as inoculum for the mechanical inoculations. In all cases carborundum (500 Mesh) was used.

One week after infection the plants were treated with either gibberellic acid (GA, 10 μg/ml) or 1 ml/l polyacrylic acid (PAA Versicol E Mwt: 230,000) in tap water. The plants were sprayed with the solutions once a week for 5 weeks, with measurement of growth taken before each treatment.

Results

Growth inhibition of virus infected plants could be observed from the second week following inoculation. The treatments increased growth in all cases. The results of the six-week-long period are summarized in the Table 1.

One can see in Table 1, that the virus infection markedly decreased the growth of diseased plants. By treatment with PAA or by GA it was possible to partially reverse the growth inhibition caused by virus infection. The effect of the two treatments in reversing the virus induced dwarfing seemed to be the same. The PAA and GA treatments affected not only the virus diseased plants, but increased the growth of the healthy ones too. Both GA and PAA acted in the same manner but the treated plants did not differ significantly in growth from the healthy control.

Table 1

The effect of polyacrylic acid of gibberellic acid treatment on the growth of healthy and tobacco etch virus-infected peppers (cv. Keystone Resistant) during a 6-week period

Treatment*	Growth (in cms)	Statistic groups**
Healthy, gibberellic acid treated	11.24	a
Healthy, polyacrylic acid treated	9.8	ab
Healthy, water sprayed control	9.3	ab
Infected, gibberellic acid treated	6.4	bc
Infected, polyacrylic acid treated	6.3	bc
Infected, water sprayed control	4.9	c

* Inoculation at 53-day-old stage, treatment one week later, GA 10 mg/l, PAA 1 ml/l (diluted in tap water).

** Treatments having the same letter do not differ significantly at the 0.05 level Duncan's Multiple Range Test

Weekly growth observations indicated that the control plants developed more or less continuously, and that the PAA or GA treatments had their major effects in first few weeks following treatment. After the 3rd treatment the plants began to reach the maximum growth, and than they developed more slowly. The infected plants were inhibited in growth and because of that the treatments had the best results in the later period. The results we got with variety California Wonder are summarized in Table 2. The virus infection also caused a strong inhibition in the development of this variety. The GA treatment enhanced the growth of infected plants, but PAA was more effective and eliminated the differences in the growth that were induced by the virus infection. The treatment increased the

Table 2

The effect of polyacrylic acid and of gibberellic acid on the growth of healthy and of tobacco etch virus infected (To 77) peppers (cv. California Wonder) during a six-week period

Treatments*	Growth (in cms)	Statistic groups**
Healthy, gibberellic acid treated	8.66	a
Healthy, polyacrylic acid treated	6.64	ab
Healthy water sprayed control	5 87	bc
Infected, polyacrylic acid treated	4.76	bc
Infected, gibberellic acid treated	3.13	cd
Infected, water sprayed control	3.0	d

* Inoculation at 100-day-old stage, first treatment 6 days later
Treatments with gibberellic acid 10 mg/l, polyacrylic acid 1 ml/l diluted in tap water
** Treatments having the same letter do not differ significantly at the 0.05 level Duncan's Multiple Range Test

Table 3

Effect of polyacrylic acid and gibberellic acid on the growth of healthy and tobacco etch virus infected (To 77) tomatoes (cv. Manalucie) during a six-week period

Treatments*	Growth (in cms)	Statistic groups**
Healthy, gibberellic acid treated	2884	a
Healthy, polyacrylic acid treated	20.08	ab
Healthy, water sprayed control	17.62	bc
Infected, polyacrylic acid treated	13.40	cd
Infected, gibberellic acid treated	12.60	cd
Infected, water sprayed control	12.20	d

* Inoculation at 43-day-old stage, first treatment 6 days later with gibberellic acid 10 mg/l, and polyacrylic acid 1 ml/l diluted in tap water
** Treatments having the same letter do not differ significantly at the 0.05 level Duncan's Multiple Range Test

development of the healthy plants was significantly greater than the water-sprayed healthy control.

Polyacrylic acid was effective in the retardation of virus induced stunting not only in case of peppers, but also in the tomato cultivar Manalucie (Table 3).

The results indicated that the tomato plants were reduced in growth after virus infection however, both treatments eliminated the significant difference between the healthy and virus infected control. The treatments acted in the same manner in the case of infected plants. The GA treatment was more effective in the healthy plants than was PAA treatment.

Discussion

Earlier experiments with polyanions and polycations demonstrated that some of them affected the replication of TMV (BURGER and STAHMANN, 1951; SHAW, 1972; STEIN and LOEBENSTEIN, 1972; ZHURAVLEV *et al.*, 1974; TYIHÁK and BALÁZS, 1976). Probably, PAA acted in the similar way and inhibited the susceptibility of hypersensitive plants to TMV infection (GIANINAZZI and KASSANIS, 1974). In our experiments PAA as well as GA were used in compatible host–parasite relation in susceptible plants, and eliminated the virus induced growth retardation. This effect of the polyacrilic acid seems to be independent from the antiviral activity because it also increased the growth of healthy plants. On the other hand, the treatment did not reduce symptom expression of the virus diseases. In our previous work (GÁBORJÁNYI *et al.*, 1973) as in the current study treatment with GA minimized the virus induced stunting, but did not reduce the severity of symptoms. Following virus infection peroxidase activity has been found to increase (SIMONS and ROSS, 1971; VAN LOON and GEELEN, 1971). In one of our earlier works (GÁBORJÁNYI *et al.*, 1973) we demonstrated a correlation between peroxidase activity and the virus induced dwarfing. By using different isolates of tobacco etch virus for inoculation of pepper, it was found that peroxidase activity correlated with the stunting effect of the virus isolate (FERNANDEZ and GÁBORJÁNYI, 1975). We supposed that the GA treatment regulating the peroxidase system could reverse the virus induced stunting. In previous experiments the GA treatment reduced the peroxidase activity of healthy and of virus infected plants but PAA did not give similar results.

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Induced Alteration of Peroxidase Activities and the Growth of Peppers Inoculated by Tobacco Etch Virus

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Three varieties of pepper (*Capsicum annuum* L.) California Wonder, True Hearth and Keystone Resistant were inoculated with seven Cuban isolates of tobacco etch virus.

The length of the infected and control plants were measured one month after the inoculation. At the same time the water soluble and the cell wall bound peroxidases were purified, separated and checked.

The severity of induced growth inhibition depended on the variety and the virus isolate used.

The peroxidase activities in the cytoplasmic fractions (soluble in cold distilled water) and the growth inhibition of the plants did not show any correlation. Negative correlation was determined between the virus induced growth and the enhanced activity of peroxidase enzymes in the cell wall bound fractions (soluble in buffer, containing sodium chloride).

Using polyacrylamide gel electrophoresis we could not detect qualitative changes in isoenzyme spectra in infected leaves compared to the control.

When the plants are infected with different fungal, bacterial or viral pathogens, generally the appearance of symptoms is accompanied by an increase of peroxidase activity (STAHMANN and DEMOREST, 1972). When the plants react with systemic symptoms on the virus infection little changes occur in the activity of peroxidases, and without any formation of new isoenzymes. However, in resistant plants when the infection is accompanied by the hypersensitive necrosis, peroxidase activity increases and often new isozymes appear (FARKAS and STAHMANN, 1966; SOLYMOSY *et al.*, 1967).

Examining the possible role of peroxidase in resistance correlation was found between the peroxidase activity and systemic acquired resistance (BOZARTH and ROSS, 1964; SIMONS and ROSS, 1970; 1971; VAN LOON, 1976) and between the increased peroxidase activity and virus multiplication (WOOD and BARBARA, 1971; VAN LOON and GEELEN, 1971).

In our earlier work we investigated the enhanced peroxidase activity induced by virus infection in connection with induced stunting. In the case of compatible host-parasite relation there was demonstrated a positive correlation between the virus induced dwarfing and increased peroxidase activities of the host stem. On the other hand, in hypersensitive plants new peroxidase isozymes appeared in the soluble and cell-wall bound fractions of inoculated leaves, but there was

no change in activity or spectra of stem fractions having importance in cell elongation (GÁBORJÁNYI *et al.*, 1973).

The aim of the present study was to determine the correlation between peroxidase activities and the growth of a systemic host, when different isolates of a given virus were applied.

Materials and Methods

Host-parasite relations. To investigate the induced growth inhibition three comercial varieties of pepper (*Capsicum annuum*, L.): California Wonder, Keystone Resistant and True Hearth were inoculated with seven isolates of tobacco etch virus originated from naturally infected tomato, pepper, *Datura stramonium* and *Portulaca oleracea* plants in the district of Havana (Cf.: FERNANDEZ, 1975: To 41; To 42; Pim 12; Pim 13; TP; Dat; Ver.). The isolates were increased in tobacco *Nicotiana tabacum* cv. Xanthi-*nc*. Two weeks after inoculation the infected tobacco leaves were harvested, and the extract diluted two fold with 0.1 *M* Sörensen phosphate buffer pH 7.1 served as inoculum for mechanical inoculations. In all cases carborundum (500 mesh) were added to the inoculum.

Peroxidases. The method of extraction and separation of peroxidases was described earlier (GÁBORJÁNYI *et al.*, 1973). Ten grams of fresh weight of leaf samples were ground with a mortar and pestle in two fold distilled water. After the centrifugation of aqueous extract (10.000 *g*/min; for 20 min) the supernatant was separated and filtrated. The pellet was suspended again in distilled water, ground in a mortar and centrifuged again. The supernatants were collected in four replications and served as "cytoplasmic soluble fraction". The residue of centrifugation was suspended in phosphate buffer (0.15 *M*; pH 7.2 containing 0.3 *M* sodium chloride), and centrifuged again. The supernatant of this centrifugation were called as "cell-wall bound" fraction (Cf.: SÁGI, 1972). All of the steps were done at 4°C.

Enzyme activities were measured spectrofotometrically according to FEHRMANN and DIAMOND (1967) with modifications, at 430 nm. The activities were caracterized as the changes of optical density in 15 sec.

The peroxidase isoenzymes were separated in polyacrylamide gels. Aliquots of 0.1 ml of dialized extract representing equal amounts of fresh weight material were put on each gel of 8.5%. 5 mA current per tube was used for three hrs. To determine the peroxidase bands the gels were incubated in solutions of saturated benzidine solution containing hidrogen peroxide. The bands were fixed in 7% acetic acid.

The measurements of growth were taken 4 weeks after the inoculation.

Results and Discussion

Growth inhibition. The seven isolates of tobacco etch virus showed differences in the severity of symptoms according to the earlier results obtained by us (CORDERO *et al.*, 1975). The infected plants produced significant differences in their growth (Table 1). We could not demonstrate any correlation between the induced dwarfing and the peroxidase activities of cytoplasmic soluble fractions. As we supposed this fraction has no important role in the plant growth.

Table 1

Comparison of the length of the three varieties of pepper inoculated with seven isolates of tobacco etch virus four weeks after inoculation

Treatment (Isolates)	Varieties					
	Keystone Resistant		True Hearth		California Wonder	
	Stem length*	Statistic groups**	Stem length*	Statistic groups**	Stem length*	Statistic groups**
Control	11.92	bc	14.72	ab	15.88	ab
TP	10.45	c	12.08	bc	15.77	ab
To 41	14.52	ab	14.12	ab	14.46	abc
To 42	10.74	c	12.89	ab	12.75	c
Pim 12	15.23	a	9.85	c	12.82	bc
Ver	11.17	c	13.92	ab	15.77	ab
Dat	12.85	abc	15.11	ab	17.35	a
	ES 1.02		ES 1.11		ES 0.84	

* Average length of 12 plants in cms

** Treatments having the same letter do not differ significantly
Duncan's Multiple Range Test

However, in fractions bound to the cell wall in the majority of the cases the peroxidases produced significant differences as compared to the control (Table 2). These differences were dependent on the severity of the given isolate.

The growth of the plants and the peroxidase activities of the cell-wall bound fractions showed a negative correlation. That means, that a severe isolate or strain of the virus attacking the plants and producing systemic stunting or dwarfing, parallelly increases the peroxidase activities too. On the other hand if the isolate or strain is mild and slightly influences the peroxidases involved in the oxidation of indolacetic acid, it will not influence markedly the normal growth of the host. The value of the correlation between the peroxidase activity and the induced dwarfing is depending on the severity of the isolate and the used variety too. For example in our experiments in the case of varieties True Hearth, California Wonder and Keystone Resistant the level of significance is 1%, 5% or higher than 5%, respectively.

On the base of Tables 1 and 2 it is possible to characterize quantitatively the severity of the isolates.

Table 2

Comparison of cell-wall bound peroxidase activities of the three pepper varieties inoculated with seven isolates of tobacco etch virus

Treatment (Isolates)	Varieties					
	Keystone Resistant		True Hearth		California Wonder	
	Peroxidase activity*	Statistic groups**	Peroxidase activity*	Statistic groups**	Peroxidase activity*	Statistic groups**
Control	3.80	a	5.10	a	32.0	ab
Tp	9.70	c	10.0	c	23.0	abc
To 41	7.65	b	10.0	c	34.0	a
To 42	10.05	c	13.5	d	33.0	a
Pim 12	7.40	b	13.5	d	27.0	abc
Pim 13	9.30	c	6.0	ab	7.5	c
Ver	7.6	b	8.5	bc	29.0	ab
Dat	7.3	b	7.0	abc	24.0	abc
	ES 3.74		ES 0.87		ES 0.03	

Averages of 3 replication. Alteration of OD in 15 seconds at 430 nm

Treatments having the same letters do not differ significantly at the level 0.5 Duncan's Multiple Range Test

Peroxidase isoenzymes. No new isoperoxidase appeared during the infection with seven isolates of tobacco etch virus as compared to the control. Only certain bands showed higher activity in accordance with the severity of the isolates we used.

In summary the peroxidase activities of cell wall bound fractions and the growth inhibition induced by systemic virus infection showed a correlation, depending on severity of the virus isolate and the host varieties. Naturally this is only one factor controling the growth of the plant.

Acknowledgement

We are gratefull to Mrs. MYRIAM ALVAREZ, Biostatistic Department of INCA, Cuba for her kind help in the statistic work.

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Trianthema monogyna L.: A New Differential Host for Tobacco Mosaic Virus Strains

By

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Trianthema monogyna was mechanically inoculated with 21 isolates/strains of TMV and they could be grouped into 7 different types on the basis of reaction and incubation periods. CMV-O–Y resulted in symptomless infection whereas TMV was characterised by production of necrotic blotches with white centres. Mixed infection of U1 was recorded in TMV-U2 lesions. 4–6 weeks plants growing at 25°C under 6,000 lux together with normal irrigation were found ideal for this host as TMV indicator. TMV-tomato strains, PVX-ring spot strain, CGMMV, SyMV-W were unable to infect this host.

For the first time *T. monogyna* was found to be susceptible to brinjal mosaic incited by a strain of cucumber mosaic virus (KHURANA, 1970). During the course of this investigation it was observed to be infected by tobacco mosaic virus-*vulgare* strain (TMV-O) to which it reacted by local lesions. But it was only in 1970–72 that further studies were undertaken, particularly with 21 strains/isolates of TMV from different parts of the world. To compare TMV with a few other, 6 more viruses were also tried to determine the diagnostic value of *T. monogyna*. Other studies like the effect of temperature, light and water, as well as interaction of two strains of TMV in this host were made. Isolates of TMV were generously donated by virologists from Brazil, Canada, Germany, India, Israel, Japan, Spain, U.K. and USA as indicated in the Table 1. All the samples were maintained in their systemic hosts kept separately in small insect-proof chambers within a glass house (25°C). Utmost care was taken to prevent any contamination of isolates among themselves or with any other virus used during the studies. TMV-U1 and U2 as well as TMV-OM were used in a purified form at a dilution of 5 μg/ml. All other viruses or strains/isolates were prepared for inoculation by macerating leaves (1 g/ml) in phosphate buffer 0.01 M, pH 7.5. The sap squeezed through cheese cloth was centrifuged once at 3,500 rpm and the supernatant recentrifuged at 12,500 rpm for 15 min each. Final supernatant was diluted (1 : 50) with the buffer for inoculation on the broad leaves of 4–6 weeks old *T. monogyna* (mainly the green ecotype), predusted with carborundum 400 mesh. *T. portulacastrum* (syn.: *T. monogyna*?) and *T. decendra* were also included in the preliminary tests.

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T. portulacastrum was found to be a local lesion host for TMV-OM, however, was not as good as *T. monogyna* in many respects. *T. decendra* was not susceptible to TMV.

Inoculated plants were maintained in a glass house for at least 30 days after inoculation, and then the symptomless plants were indexed for the inoculated virus on its respective indicator. Symptom descriptions are based on 3–5 tests with 5 replicates/virus or strain in each test.

To determine the effect of temperature, phytotron chambers maintained at 15, 20, 25, 30 and 33°C were used to grow *T. monogyna* plants at least 2 weeks before and up to 4 weeks after inoculation with TMV-OM. The effect of light intensity on the susceptibility and reaction of *T. monogyna* to TMV-OM was observed on plants maintained in phytotron chambers at 25°C having different photo-intensities, viz., 2,000, 4,000, 6,000 and 10,000 lux.

T. monogyna responded to virus infections differently depending on the virus, however, some of the isolates did not differ much in symptoms but the time taken

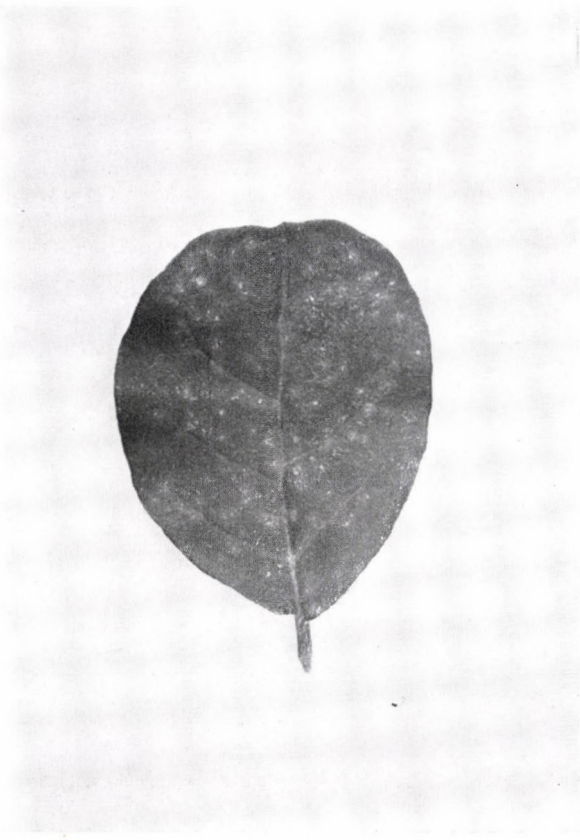

Fig. 1. *T. monogyna* leaf showing local lesions (pin prick spots) incited by TMV-U1

for symptom development of infection varied (Table 1). All the TMV strains, excepting 10 strains/isolates from tomato, produced local lesions (Figs 1, 2). In addition to TMV, a Japanese strain of TNV induced irregular lesions like white blotches (Fig. 3). Two strains of CMV(O,Y) developed systemic infection but the

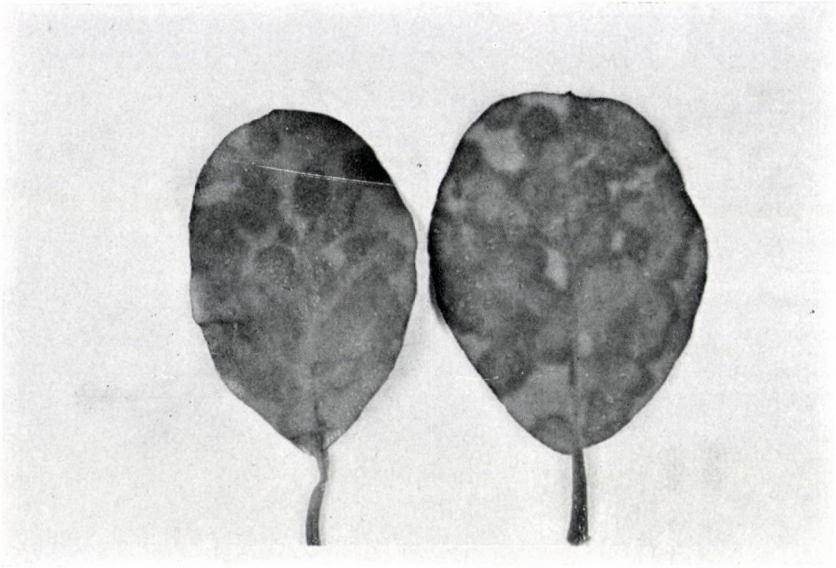

Fig. 2. *T. monogyna* leaves showing typical local lesions (yellow, coalescing spots) incited by TMV-OM

Fig. 3. *T. monogyna* leaves exhibiting irregular, white centred lesions (blotches) incited by TNV

Table 1

Reaction of *T. monogyna* to different strains of TMV and six other viruses

Virus (Strain/isolate)	Source-host	Reaction	I. P. (days)	Avg. no. lesions/ leaf	TMV-T.m. Group	Donor
TMV-U1	*N. t.* H. S.	LL (Pin-prick spots)	4	45	I	W. C. Wetter, Germany
TMV-BM	*B. diffusa*	—	—	—		S. Singh, India
	N. t. Turkish	LL (Pin-prick spots)	5	36	I	
TMV-O	*N. t.* Turkish	LL (Pin-prick spots)	4	32	I	S. Singh, India
TMV-*Vulgare*	*N. t.* H. S.	LL (Pin-prick spots)	4	39	I	K. S. Bhargava, India
TMV-U2	*N. t.* H. S.	LL (Faint yellow spots and withering leaves)	11	16	II	P. C. Cheo, U.S.A.
TMV-OM	*N. t.* BY	LL (Big yellow spots)	8	43	IIa	KyU, Japan
TMV-PP	*N. t.* BY	LL (Dark-Yellow spots)	3	11	III	V. S. Verma, India
TMV-Brazil	*N. t.* BY	LL (Pin-pricks with chlorotic halo)	6	28	IV	K. Silberschmidt, Brazil
TMV-Israel	*N. t.* Samsun	LL (Light-yellow spots)	8	13	V	G. Loebenstein, Israel
TMV-DT	*N. t.* Turkish	LL (with violet periphery and 2—3 conc. rings)	3	11	VI	M. Rubio Huertos, Spain
TMV-ACRI	*N. t.* Turkish	LL with violet halo	8	19	VIa	T. K. Kandaswami, India
TMV-Tm(6)[b]	Tomato	—	—	—	VII	J. T. Fletcher, UK
TMV-Tm(3)[c]	Tomato	—	—	—	VII	Y. Ohtsu, Japan
TMV-Tm(1)[d]	Tomato	—	—	—	VII	B. H. MacNeil, Canada
TNV	*Ch. am.*	LL irregular, white centres and violet periphery (with or without a halo)	4	11		KyU, Japan

CMV-O	*C. sativus*	Symptomless	15	6		KyU, Japan
CMV-Y	*C. sativus*	Symptomless	21	—		KyU, Japan
CMV-W	*C. sativus*	—	—	—		KyU, Japan
CGMMV-Wm	*C. sativus*	—	—	—	—	KyU, Japan
PVX-RS	*N. rustica*	—	—	—	—	KyU, Japan
SyMV-necr.	Soybean	—	—	—	—	KyU, Japan
SuMV-Delhi	*C. juncea*	— (Syst. ? 1/5×3 plants)	—	—	—	K. Kartha, India

I. P. — Incubation period, LL — Local lesions, TMV-T.m. group — TMV type (group) according to *T. monogyna* reaction, *N. t.* H. S.—*N. tabacum* var. Harrison Special, *B. diffusa* — *Boerhaavia diffusa*, *N. t.* Turkish-*N. tabacum* var. Turkish, *N. t.* Samsun- *N. tabacum* var. Samsun, *N. t.* BY—*N. tabacum* var. Bright Yellow, *Ch. am.*— *Chenopodium amaranticolor*, *C. sativus*— *Cucumis sativus*, *C. juncea* — *Crotalaria juncea*, KyU — Kyushu University, Fukuoa, Japan.

TMV strains/isolates: BM — *Boerhaavia* mosaic, PP — Potato 'Phulwa', DT — *Digitalis thapsi*, ACRI — Agric. Coll. & Res. Inst., Coimbatore, OM — Ordinary mosaic, Tm — Tomato strains.

a — Apparently a variant of the group.

b — TMV-Tm — O(F35/A; 33/4/AB/AF), Tm-1 (F 21/SA) Tm-1 : 2 (F 49/A; F 21/SA) Tm-2 (33/4/AP/A)

c — TMV-Tm-O (Three distinct, yet unnamed, isolates from Japan)

d — TMV-Tm-O (F 352 – 6).

symptoms were not marked, i.e., *T. monogyna* is symptomless carrier for these. CMV-W, soybean mosaic virus-necrotic strain (SyMV-necr.), potato virus X-ring spot strain (PVX-RS) and cucumber green mottle mosaic virus-watermelon strain (CGMMV-Wm) did not produce any infection in *T. monogyna*. Of the five trials, only once one particular replicate was found to have developed symptomless infection of the Delhi(?) strain of sunnhemp mosaic virus (SuMV-D). The infection of *T. monogyna* by SuMV-P could not be repeated, however.

Effect of temperature

Groups of 10 plants each, maintained separately at 5 different temperatures but receiving the uniform light of 6,000 lux, reacted in three ways. Plants maintained at 15°C developed general chlorosis and stunting but no local lesions. Plants maintained at 20°C, 25°C and 30°C exhibited only local lesions on TMV-OM infection. The fifth group at 33°C developed only 15–20% lesions (as against control at 25°C) and took 10 days as incubation period; however, some replicates also developed systemic infection as confirmed by back inoculation from uninoculated upper leaves.

It was interesting to note that *T. monogyna* plants which were transferred from 15°C either 4 or 7 days after inoculation to 25°C reacted differently. When plants were transferred 4 days after inoculation they developed local lesions within next three days of incubation at 25°C, whereas plants after 7 days at 15°C on transfer to 25°C did not develop lesions.

Effect of light

T. monogyna plants maintained at 25°C but under varying light intensities on infection with TMV-OM revealed that maximum number of lesions (avg. 43/leaf) were developed within eight days under 6,000 lux condition. The number of lesions decreased and incubation period was prolonged by about 15% and 2 days, respectively, under both 4,000 and 10,000 lux treatments. Plants maintained at 2,000 lux did not produce lesions but developed systemic infection. In about 30–35 days these plants exhibited marked stunting and general chlorosis accompanied with slight cripling of axillary leaves.

Effect of water availability to plants

Three groups of 5 plants each when subjected to excess, moderate or less irrigation, only the latter two groups developed usual number of lesions per leaf within the minimum period of incubation. Plants supplied with excessive irrigation were found to be less susceptible to TMV infection and exhibited symptoms only after prolonged incubation period.

Interaction between two strains of TMV in T. monogyna

TMV-U1 and -U2 (purified preparations) when inoculated in successive orders on same leaves the period of incubation for lesion development was reduced

for U2 by 3 days if U1 succeeded 24 h or 48 h after U2 inoculation. When U2 succeeded U1 by 24 h but not 48 h, the U2 lesions were also developed within 2 days after appearance of U1 lesions, i.e., U1 infection has a negative interaction meaning thereby it hastens U2 multiplication, as well.

The simultaneous inoculation of U1 + U2 resulted not only in early development of U2 lesions but also that 40% lesions had mixed infection of U1. In fact U1 lesions were developed within U2 lesions (Fig. 4).

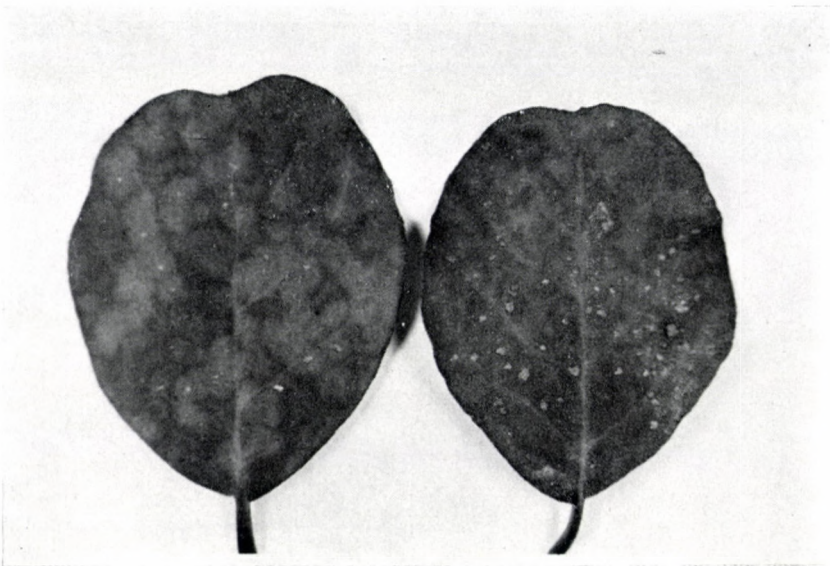

Fig. 4. *T. monogyna* leaves inoculated with mixed inocula of TMV-U1 and -U2 showing U1 pin prick spots within the U2 lesions

The reactions of six viruses are tabulated; 21 isolates/strains of TMV from different parts of the World could be grouped in 7 types on the basis of their reaction. TMV could be very well distinguished from TNV and other four viruses did not infect, *T. monogyna* which was found to be a symptomless host of CMV strains also.

It is therefore endorsed that *T. monogyna* can be safely used for preliminary diagnosis of TMV to strain level. Of interest is the fact that simultaneous infection of TMV-U1 and -U2 resulted in not only single lesions being formed by two strains of a virus but also reduction in incubation period for U2. This is the second record of such lesions having mixed infection casting doubts on the reliability of the single lesion isolation of viruses from mixtures. KULKARNI (1970) evidenced for the first time mixed infection of two viruses of papaya within single lesion.

Acknowledgements

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The Role of *Tetranychus telarius* L. and *T. atlanticus* Mc Gregor *(Acarina: Tetranychidae)* in the Transmission of Paprika Viruses

By

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The question whether *Tetranychus telarius* and *T. atlanticus* is able to transmit the viruses of paprika under glasshouse conditions has been investigated. It was concluded that these two spider mite species cannot transmit the tobacco mosaic virus (TMV-U_1), cucumber mosaic virus (CMV-W), alfalfa mosaic virus (AMV-L), potato aucuba mosaic virus (PAMV), and potato virus Y (PVY).

Introduction

The virus diseases of paprika caused the most important losses of paprika production both in the field and in glasshouses during the last years in Hungary (Kuroli, 1971; Kajati and Kádár, 1974): The viruses in the glasshouse can become even more important than in the field since the pathogens are spread in the closed environment by virus vectors throughout the whole plant stand.

From the three most important groups of pests in the glasshouses: the aphids, the white flies, and the spider mites, the two first ones are already known as possible virus vectors, while the informations available in this respect for the spider mites are scarce and contradictory.

Schulz (1963) showed that *Tetranychus telarius* is able to transmit potato virus Y. However, Orlob (1968) investigated the virus vector role of *Tetranychus telarius* in the case of 9 viruses, among others also with the potato virus Y, and found no successful transmission. In further experiments of Orlob and Takahashi (1970, 1971), *T. urticae* failed to transmit tobacco ringspot and brome mosaic virus.

Material and Method

The experiments were carried out in 1973 and 1974 in glasshouses. The paprika variety "Javított Cecei" was used for the tests carried out with females

* The authors express their gratitude to Dr. J. Horváth, senior plant pathologist of the Research Institute for Plant Protection, who kindly provided virus infested material for the experiments.

of *T. telarius* and *T. atlanticus*. The following viruses* were included into the tests:

cucumber mosaic virus (CMV-W),
alfalfa mosaic virus (AMV-L),
potato aucuba mosaic virus (PAMV) (Fig. 1),
tobacco mosaic virus (TMV-U_1),
potato virus Y (PVY).

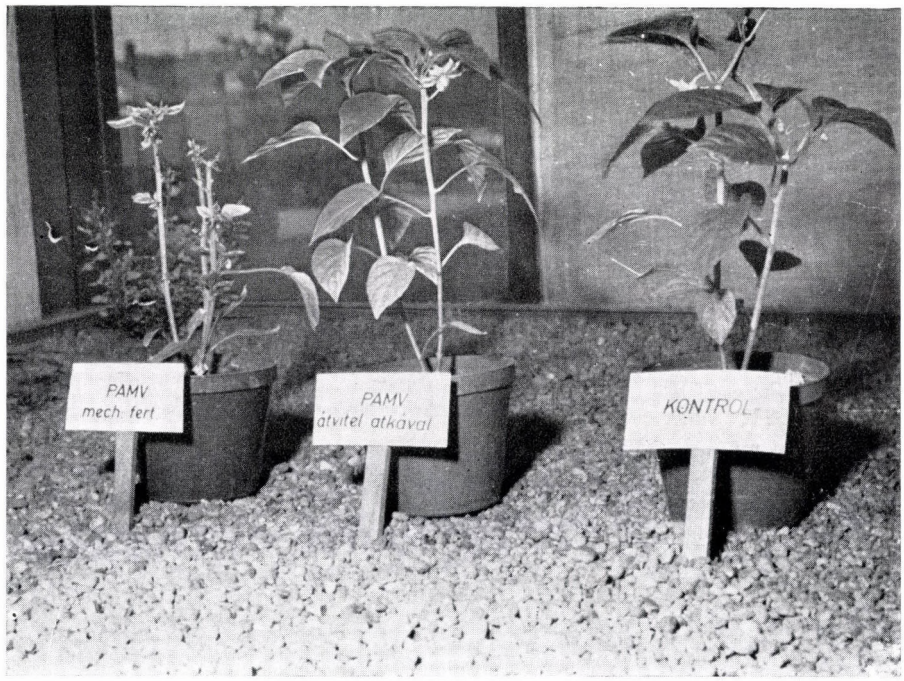

Fig. 1. Left: PAMV-infected paprika plant (mechanical infection). Middle: The virus transmitted by spider mites. Right: uninfected (control) plant

The paprika plants *Capsicum annuum* grown from virus free seeds were inoculated with one of the above viruses at the six leaves stage. After the appearance of symptoms all leaves were removed except one infected leaf, and 10 female spider mites were transferred onto it by a fine brush. After 48 hours of feeding the females were transferred on healthy plants and the latter were surveyed for the appearence of symptoms during one month. For each virus 40 plants and 400 spider mites were used, while 10 plants with 100 spider mites served as a control.

The main data of the microclimate in the glasshouse during the experiments were the following:

Relative air humidity (mean): 76%

Temperature:

Average of the whole period	18.9°C
Average of daily maxima	27.7°C
Highest maximum	34.0°C
Average of daily minima	11.8°C
Lowest minimum	7.0°C

Daily illumination: 16 hours

Results and Discussion

No transmission of any of the above mentioned viruses by the spider mites from the infected to the healthy plants could be observed. However, it has to be emphasized that the statement on the failure of the transmission is based only on the lack of the appearance of symptoms, since the latent infection of the viruses has not been determined.

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Laboratory Experiments with Juvenoids on the San José Scale, *Quadraspidiotus perniciosus* Comst.

By

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Six juvenoids, SJ-53, ZR-512, ZR-515, ZR-619, ZR-777, and R-20 458 were applied in 0.001—1% acetone solutions or aqueous emulsions to young apples and to shoots of red currant infested by the San José scale. Second instar larvae and pronymphs (prepupae) were treated in both the overwintered and the summer generations. The JH analogues in 0.01% or 0.1% concentrations induced relatively high levels of mortality in males but only moderate mortality in females. Males died mostly as nymphs (pupae), females as young, unproductive adults. In the low concentrations, the natural hymenopteran parasitoids of *Quadraspidiotus* seemed not to be affected by the juvenoids. In the highest concentration of 1% which proved effective in both sexes of the host, the emergence of parasitoids was significantly reduced. A suggestion for rational and economic control of the San José scale was put forward: selective juvenoids can be successfully used as control agents against the male population of the overwintered generation in pronymphal and nymphal stages.

The considerable economic and quarantine significance of the San José scale as a notorious pest of some fruit species in Hungary has necessitated intensive and regular use of potent insecticides. For integrated plant protection in which reduction of sprayings and more effective use of chemical treatments are required, suitable measures for scale insect control should also be re-examined. As the result of a detailed investigation of the insecticide-sensitive developmental phases and the life-cycle of this species, more rational and economic control methods against *Quadraspidiotus* have been suggested (SHETA, 1968). Nevertheless, the application of conventional insecticides has still left some questions unsolved (JENSER and SHETA, 1972; KOZÁR, 1975). This fact stresses the importance of searching for selective control methods and agents in this field of practice.

BAGLEY and BAUERNFEID (1972) were first to suggest the application of juvenile hormone analogues to scale insects. The use of insect growth regulators (e.g. juvenoids) as control chemicals against this group of pests is considered perspective since – as in other Homoptera – the occurrence of economically important damage generally requires a population buildup through several generations (STAAL, 1975). The synthesis of some highly active juvenoids and their administration to scale insects has brought this assumption close to reality.

In the family of soft scales *(Coccidae)*, positive results were published by BAGLEY and BAUERNFEIND (1972), SCHEURER and RUZETTE (1974), and HAMLEN

(1975a, 1975b). In mealybugs *(Pseudococcidae)*, members of a related family, similar promising data were reported (STAAL *et al.*, 1973; HAMLEN, 1975a). Concerning armored scales *(Diaspididae)*, BAGLEY and BAUERNFEIND (1972) tested JH analogues on *Unaspis citri*. Detailed results were reported on *Hemiberlesia lataniae* by NASSAR *et al.* (1972), on *Aspidiotus nerii* by SCHEURER and RUZETTE (1974), as well as on *Aonidiella aurantii* by BOBOYE and CARMAN (1975). On the most important diaspidid in Europe, the San José scale, VOGEL and HANGARTNER (1974) conducted an experiment in which the symptoms of juvenoid action in consecutive developmental stages and the changes in JH sensitivity were thoroughly studied.

In our laboratory investigations, performed on natural populations o *Quadraspidiotus perniciosus*, we aimed to study the developmental effects of juvenile hormone analogues and the efficiency of using them as insecticides also noting the action on parasitoids.

Materials and Methods

Prior to treatments, young apples and shoots of red currant infested by natural populations of the San José scale were collected in the field (Csopak near Lake Balaton, Pomáz near Budapest). Applications of juvenoids were carried out on the overwintered generation (April 23, 1974) and on the summer generation (July 12, 1973). Insects were treated when the majority of individuals reached the second larval instar or the pronymphal (prepupal) stage.

The surface of the apples and the shoots of red currant were treated by dipping them into aqueous emulsions of different juvenoids or by painting them with acetone solutions of chemicals. The following compounds were applied:

ethyl-11-chloro-3,7,11-trimethyl-2-dodecenoate (SJ-53; Institute of Organic Chemistry, and Biochemistry; Czechoslov. Acad. of Sci.);
ethyl-3,7,11-trimethyl-2,4-dodecadienoate (ZR-512; Hydroprene, Altozar; Zoecon Corp.);
isopropyl-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate (ZR-515, Methoprene, Altosid; Zoecon Corp.);
S-ethyl-11-methoxy-3,7,11-trimethyl-2,4-dodecadienethioate (ZR-619, Triprene; Zoecon Corp.);
2-propynyl-3,7,11-trimethyl-2,4-dodecadienoate (ZR-777, Kinoprene, Enstar; Zoecon Corp.);
1-(4'-ethyl-phenoxy)-6,7-epoxy-3,7-dimethyl-2-octene (R-20 458; Stauffer Chem. Co.).

The concentrations of juvenile hormone analogues ranged from 0.01% to 1%. In some cases 0.001% emulsions were also tested. Control groups received only water. In the trial with replicates 4 parallel groups were set up. After treat-

ment, the apples and the shoots (placed into jars with water) were preserved at 20 to 25°C.

Mortalities of various instars in both sexes were recorded. A certain number of scale coverings (scutes) was opened and the insects were observed under binocular stereo-microscope. Checks were made 7, 14, 18 days and 2 to 3 months after treatment. The rate of parasitoid emergence was also calculated. For microscopic investigations special preparations were made.

Results

In a trial conducted on the summer generation of the San José scale, 4 juvenile hormone analogues were administered to infested apples, two of which were in emulsions. Results received in two checks are summarized in Table 1. In the controls only males exhibited high "natural" mortality after 18 days but the percentage of mortality was raised considerably in treated groups. This enhanced mortality was already well expressed a week after application of juvenoids and changed, in general, within the next 11 days very insignificantly. As a rule, the greater ratios of mortality were in good accordance with the higher concentrations applied.

All of the four compounds proved effective on males. Treatments with 0.01% or 0.1% solutions and emulsions of juvenoids resulted in significantly increased (up to 100%) mortality. The JH analogues in 1% concentration killed the whole male population within 18 days. Female individuals exhibited much lower sensitivity to juvenoids than males. In this sex only 1% solutions or emulsions could evoke higher mortality rates.

Fine distinctions concerning JH activities between individual juvenoids could not be made here. In the cases of ZR-512 and SJ-53 the aqueous emulsions seemed more effective than acetone solutions, especially on females.

In the final check (2 months after treatment) all insects remaining on apples were thoroughly investigated. We determined the terminal developmental forms into which the control and treated scale insects developed. In this way we could also ascertain the critical developmental phases in which the highest mortalities occurred. Data concerning male individuals are found in Table 2 and those referring to females in Table 3.

Males died mostly as nymphs (pupae). These insects attempted to moult but were unable to shed the old skin, especially on the abdomen. Inspections of microscopic preparations revealed similar deficiencies at ecdyses from L_2 to pronymph (prepupa) and pronymph to nymph but they were found at much lower frequencies. Such JH affected forms, strapped by the former cuticula, are demonstrated in Fig. 1. A few males also died before emergence from the scutes.

As a consequence of juvenoid treatments, most females were killed in an early adult stage. Only a small number produced crawlers before death.

Table 1

Mortality of the San José scale induced by juvenoids (summer generation on young apples)

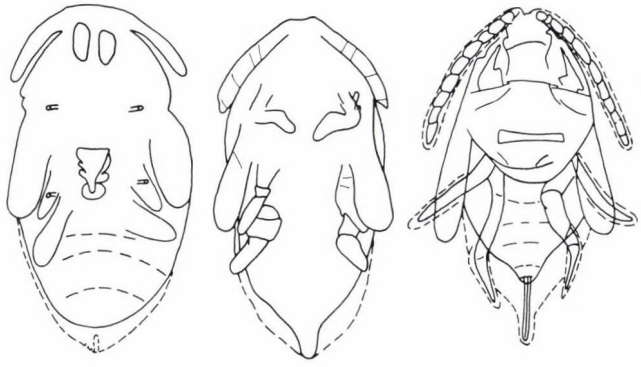
Compound	Application*	Conc.: (%)	Mortality of males (%)							
			0		0.01		0.1		1	
		Check:*	1	2	1	2	1	2	1	2
Control	(water treated)		17	70						
SJ-53	AC				70	91	73	87	86	100
	EM				42	100	100	100	92	87
ZR-512	AC				57	70	67	87	65	100
	EM				33	70	77	93	100	100
ZR-515	AC				70	82	50	93	87	100
R-20458	AC				33	87	67	93	100	100

Compound	Application*	Conc.: (%)	Mortality of females (%)							
			0		0.01		0.1		1	
		Check.:*	1	2	1	2	1	2	1	2
Control	(water treated)		27	20						
SJ-53	AC				0	27	0	33	33	27
	EM				0	33	20	10	60	93
ZR-512	AC				13	7	7	53	25	7
	EM				13	7	7	20	93	80
ZR-515	AC				0	13	0	13	100	100
R-20458	AC				0	0	13	60	80	80

+ Application: AC = acetone solution
EM = aqueous emulsion

* Checks: 1 = 7 days after treatment
2 = 18 days after treatment

In both cases 15 males and 15 females were checked in each experimental group



1 2 3

Fig. 1. Dead 2nd instar larva (1), pronymph (2), and nymph (3) of the San José scale with moulting defficiencies evoked by juvenoids. ——— = new skin; - - - - = old skin

Table 2

Influence of juvenoids on the development of males in the San José scale (summer generation on young apples)

Compound	Application+	Concentration (%)	No. of indiv.	Percent of terminal developmental forms			
				pro-nymphs	nymphs	preemergence adults	normal adults
Control	(water treated)	0	58	8	45	7	40
SJ-53	AC	0.01	20	0	85	0	15
		0.1	40	0	85	0	15
		1	16	6	63	0	31
	EM	1	52	0	85	6	9
ZR-512	AC	0.01	13	0	77	0	23
		0.1	44	0	91	0	9
		1	37	0	87	5	8
	EM	0.01	40	0	88	0	12
		0.1	46	0	37	2	11
		1	22	13	77	5	5
ZR-515	AC	0.1	33	0	80	0	20
R-20458	AC	0.01	27	0	82	0	18
		0.1	24	4 y	75	0	21
		1	24	0	85	4	13

+ See foot-note of Table 1

Table 3

Influence of juvenoids on the development of females in the San José scale (summer generation on young apples)

Compound	Application+	Concentration (%)	No. of indiv.	Percent of terminal developmental forms			
				dead unproductive	dead productive	living productive	parasitized
Control	(water treated)	0	25	16	16	60	8
SJ-53	AC	0.01	52	65	0	0	35
		0.1	59	59	0	29	12
		1	19	47	0	37	16
	EM	0.01	72	60	3	1	36
		0.1	46	33	2	48	17
		1	46	98	0	2	0
ZR-512	AC	0.01	122	43	10	17	30
		0.1	33	49	0	12	39
		1	13	31	0	46	23
	EM	0.01	73	68	0	10	22
		0.1	140	83	3	8	6
		1	34	97	3	0	0
ZR-515	AC	0.01	46	15	2	20	63
		0.1	52	71	0	8	21
R-20458	AC	0.01	43	44	0	40	16
		0.1	52	94	0	0	6
		1	38	97	0	3	0

+ See foot-note of Table 1

Table 4

Mortality of the San José induced by juvenoids (overwintered generation on shoots of red currant)

Sex	Concentration (%)	Mortality %*				
		Control	SJ-53	ZR-512	ZR-619	ZR-777
Male	0	42 (58) a				
	0.001				78 (48) b	63 (62) a
	0.01		63 (50) a	59 (114) a	66 (78) a	61 (36) a
	0.1		51 (68) a	83 (32) b	79 (60) b	89 (69) b
	1		90 (132) b	100 (56) c		
Female	0	2 (239) a				
	0.001				40 (112) d	28 (158) b
	0.01		27 (164) b	23 (192) b	38 (120) d	39 (163) d
	0.1		77 (184) c	74 (122) c	46 (123) d	30 (175) b
	1		87 (151) c	80 (150) c		

* Mean of 4 replicates; in parentheses = No. of individuals checked 14 days after treatment. Between means followed by the same letters (in each sex) no significant differences ($p = 0.10$) were found

In another experiment, 4 juvenoids were used against a *Quadraspidiotus* population that overwintered on shoots of red currant in the field. The emulsions of JH analogues were applied when the first pronymphs were found. The average percentages of mortality determined in a check 14 days after treatment are illustrated in Table 4. Here again, two compounds, SJ-53 and ZR-512 acted fairly well in 1% concentration. The other two chemicals, especially recommended against *Homoptera*, ZR-619 and ZR-777 significantly influenced the development of the San José scale in 0.1% emulsions. The higher sensitivity of male insects was also observed in this trial.

Some information on the influence of juvenoids on the development of parasitoids infesting *Quadraspidiotus* was gathered. Two hymenopteran species, *Prospaltella perniciosi* Tow. and *Aphytis proclia* Walk. parasitized the experimental animals. They occured in ratio 98 : 2. The juvenile hormone analogues in 0.01% and occasionally in 0.1% did not seem to alter the level of parasitism (Table 3). After application of 0.01% solutions and emulsions, high rates of parasitoid emergence were usually observed. On the other hand, it was quite obvious that the 1% concentrations of juvenoids decreased the percentage of parasitized females, sometimes down to 0%.

Discussion

Experimentation on the San José scale in laboratory is often accompanied by serious methodical problems. The fruits or shoots of host plants, collected in the field, can not always be well preserved. This fact may influence the survival of pest. Infestation on different plant samples is generally unequal and this varia-

tion in population density could also have some effect on mortality rates. Therefore, under laboratory conditions, we have to deal with relatively high percentage of "natural" mortality with large standard errors in the means.

The distinct delay in expression of developmental disturbances shows that we can indeed observe typical hormonal effects. After applying higher dosages, however, one can not exclude the possibility of insecticidal action. The immediate or rapid block of development, found also by BOBOYE and CARMAN (1975), was ascribed to a direct acute toxicity.

In our experiments, juvenoids of different chemical structures, used in 0.1% concentration, provided satisfying control against males of the San José scale. After treatments in the second larval instar or the pronymphal stage, the developmental block in males occurred mostly at the time of moulting, especially at the nymph-adult ecdysis. In *Quadraspidiotus perniciosus*, enhanced JH-sensitivity of males during metamorphic moults was also reported by VOGEL and HANGARTNER (1974). Males of other armored scales also proved vulnerable to juvenile hormone analogues (NASSAR *et al.*, 1972; SCHEURER and RUZETTE, 1974; BOBOYE and CARMAN, 1975). We must add, however, that during transformation from pronymph to adult the "natural" mortality of males may also be high (see Table 1 and 4) and, as JENSER and SHETA (1972) observed, the insecticide-sensitivity is also raised.

In females the JH analogues evoked generally much lower rates of mortality than in males. In this sex only 1% concentration gave promising results. Female individuals died mostly as young, unproductive adults. We may assume that these insects were not inseminated since appropriate numbers of males could not be present.

The difference in JH-sensitivity between sexes may attributed to the greater size of females or to the longer time interval between treatment and the main period of imaginal differentiation that ensues later in females than males. It is more probable, however, that the two additional moults accompanying metamorphosis, typical for diaspidid scales, render the males more sensitive to juvenile hormone analogues.

We did not find any signs of supernumerary moults in JH-treated scale insects which is in good accordance with the results of BAGLEY and BAUERNFEIND (1972) as well as BOBOYE and CARMAN (1975). We have never seen any "supernymphs" described by VOGEL and HANGARTNER (1974). Other types of developmental abnormalities or defects in scale formation could not be detected.

Our laboratory experiments did not provide an opportunity to discriminate between various juvenoids concerning their JH activity. The compound R-20458, possibly the most effective on *Aonidiella* (BOBOYE and CARMAN, 1975) as well as ZR-619 and ZR-777, active on *Hemiberlesia* (NASSAR *et al.*, 1972) exhibited good efficiencies, especially on males, in our experiments. At present we can not explain why two chemicals, ZR-512 and SJ-53, were more active in aqueous emulsions than in acetone solutions.

The juvenoids in 0.1% or lower concentrations did not prove disadvant-

ageous against *Prospaltella perniciosi* and *Aphytis proclia*, the two main hymenopteran parasitoids of *Quadraspidiotus*. The highest concentration used (1%), however, was generally detrimental for these species. SCHEURER and RUZETTE (1974) reported no adverse influence of juvenoids to *Prospaltella perniciosi*. On the other hand, HAMLEN (1975b) found an inhibitory action of JH analogues on a parasitoid, *Encyrtus infelix*, infesting the hemispherical scale.

It would be difficult to find the reason for the decreased emergence of parasitoids from JH-treated scale insects. One can imagine a direct hormonal effect on the parasite but also an indirect one which exerts its action via the host. We hope that with the use of selective juvenoids, the unwanted mortality of parasitoids or the reduction of parasitism in *Quadraspidiotus* can be avoided.

For replacing present and not always economic and effective methods in the control of the San José scale with new measures, sprayings in periods of nearly synchronous development in population were suggested. Hence, applications of insecticides should be limited to spring (April-May) when males pass the metamorphic moults and swarm or to early summer (June) when productive females as well as crawlers and settled first instar larvae are found (SHETA, 1968; KOZÁR, 1975). Since the mass-production of crawlers often or regularly coincides with the ripening of some stone fruits, the possibility of male control seems more feasible (JENSER and SHETA, 1972). Swarming males in each fruit cultivation can be well attacked with insecticides (KOZÁR, 1975). However, still more effective control might be expected if pronymphs and nymphs could be also killed. This way the insemination of females is actually well inhibited (JENSER and SHETA, 1972). In the case of the latter possibility, however, we have another serious difficulty, namely, that these sprayings at flowering time endanger pollinators. This is the problem, for example, with the apple which is economically the most important host of the San José scale in Hungary.

In conclusion, we propose the use of juvenile hormone analogues – in possible low concentrations – against the most sensitive and relatively synchronously developping forms of the San José scale, i.e. males of the overwintered generation in pronymphal and nymphal stages. In any case, it is a very important pre-condition for appyling juvenoids in orchards that the compound should be highly selective having no adverse effects on bees, parasites and other beneficial insects.

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Zur Kenntnis der Insektenfauna und ihre Krankheiten in einigen alten Mühlen des Kosova Gebietes, Jugoslawien

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In course of regular surveys carried out in 11 old mills in the mountain area of Kosovo County, Jugoslavia, the author established the occurrence of 8 pests of stored products, the population densities of which were influenced by 6 predators, 5 parasites and 12 pathogens. From the latter the pathogens *Mattesia dispora* Naville, *Farinocystis tribolii* Weiser and *Adelina tribolii* Bhatia are described to more details, with regard to their morphology and the disease symptoms caused in their hosts. It is of special interest that these studies on the host-parasite relationships were made in localities where chemical insecticides were never used, so the density regulating factors acted under more or less natural conditions.

Die schnelle Umgestaltung der Landwirtschaft in Europa im Laufe der letzten 30 Jahren verwischte das ursprüngliche Bild des Auftretens von Vorratschädlingen in isolierten Herden und der ursprünglichen Beziehung zwischen Region, Typ des Herdes und seines Schädlings inventars ihrer Feinde und Krankheiten. Trotzdem wäre ein Studium dieser Umstände und die Herausbildung eines biologischen Gleichgewichtes in ungestörten, durch Insektizide nicht behandelten Fundorten sehr wichtig, auch bezüglich eventuell neuer verfahren zur biologischen Bekämpfung der Vorratschädlinge.

Während dieser Studie über die Verbreitung verschiedner Insekten in dem Kosova-Gebiet von Jugoslawien zeigte sich ein Möglichkeit mehr Auskünfte zu dieser Frage zu sammeln, da in diesem Gebiet noch Dorfmühlen erhalten blieben, die im gebirgigen Gelände und wegen den schwierigen Umstäden noch immer winzige Dorfgemeinden versorgen und fast keine Verbindung mit der Hauptstrassen der Getreide- und Mühlwaren-Versorgung haben. Auch ist in diesen Dorfmühlen die Behandlung mit Insektiziden unbekannt. Deswegen wurden einige solche Mühlen besucht und Material zur Bestimmung der dort vorhandenen Schädlinge gesammelt.

Heute bilden die alten Mühlen in Kosova ein Netz kleiner Betriebe, die ihre geschlossenen Versorgungsgebiete haben, meist nicht weiter als 10 bis 15 km im Unkreis. Sie liegen entlang kleiner Flüsse in wilden Kluften und engen Tälern ausgedehnter Gebiergsmassive. Meistens lassen sie sich im westlichen Teil des Kosova-Gebietes, am Flusse Bistrica e Pejës, Prizrenit, Deqanit (Peéska, Prizrenska, Deqanska Bistrica) und anderen Flüsse finden. Diese Mühlen dienten

jahrzehntelang zum Mahlen von Weizen und Mais, nur selten von Gerste oder Roggen. Da, der Müller den Vorliebe einzelner Bauer für bestimmte Typen von Mehl ohne weiteres entgegenkommt, sind die Mühlen noch immer gesucht. Doch es verringert sich von Jahr zu Jahr die Menge des hier gemahlenen Getreides da immer mehr Getreide in den staatlichen Geschäften gekauft, ohne Strappazen der Aufbewahrung und Handhabung der Mühlprodukte auf der Farm.

Die Mühlen können in etwa drei Gruppen eingeteilt werden. Die ganz kleinen haben nur ein oder zwei Mühlsteine, mit einer Tagesproduktion von 200 bis 300 kg Mehl, mahlen sie meistens nur etwa 6 Monate und dann werden sie abgestellt da auch kein Wasser mehr zu haben ist. In der zweiten bzw. dritten Gruppe sind Mühlen mit 3 oder 4 vorhanden Steinen, mit einer Tagesproduktion von 500 bis 700 kg. Bei diesem klienen Unfange des gemahlenen Getreides ist es klar, dass nicht viel Mehl in den Mühlen gelagert bleibt, es sind meistens nur 300 bis 400 kg täglich und eine Zeit lang bleibt die Mühle ausser Betrieb, wodurch auch die Möglichkeit kontinuirlicher Erhaltung der Schädlinge verringert wird. Bei der primitiven Konstruktion der Mühlwerke gibt es nur wenige Verstecke wo Überreste der Mühlprodukte verbleiben und den Schädlingen Boden zum Überleben bieten nicht so wie die Mühlsteine, wo Mehlresste längere Zeit hindurch erhaltenbleiben von wo auch das Material zu dieser Studie genommen wurde.

In diesen Mühlen werden keine chemischen Mittel benutzt und jeder Eingriff dieser Art wäre sehr schwierig, da die Bauten zur ausgiebigen chemischen Behandlung ungeeignet sind. Sie sind aus Stein und Holz gebaut und in ihren vielen Fugen finden die Schädlinge überall Zuflucht.

Beschreibung der untersuchen Mühlen

Shtimle: Eine kleine Mühle mit einem Mühlstein in einem Gebirgsort in Kosova. Sie arbeitet unregelmässig je nach dem Wasserstand, hat eine geschlossene Klientelle im allernächsten Umkreis.

Radavci: Ein Dorf am Abhang eines grossen Gebirgsmassivs (Mokra, Zhlebi) mit drei Mühlen (2 mit je 3 Mühlsteinen und ein mit 4 Steinen). Alle drei haben beständig Wasser zur Verfügung und sind im ganzen Jahr tätig. Alle drei stellen einstöckige Gebäude aus Stein und Holz dar und befinden sich am Ufer des Drini i bardhe (Weisses Drim). Die Menge des gemahlenen Getreides ist ziemlich gross, über 100 000 kg im Jahre. Sie enthalten eine reichliche Schädlingsfauna mit Parasiten und Pathogenen.

Carrabregu, Isniqi: In diesen Dörfern sind sieben Mühlen vorhanden, die Häuser aus Stein und Holz gebaut und haben mehrere Etagen. Im Erdgeschlosse ist die Mühle, oben die Wohung des Müllers. Sie bedienen meistens die Dorfbewohner in der Saison und Mahlen kleine Getreidemengen. Sie stellen geschlossene Biotope dar und enthalten eine beständige Schädlingsfauna samt ihren Parasiten und Krankheiten.

Dubova, Kashica, Tekuli: Dörfer in tiefen, fruchtbaren Tälern, vorwiegend mit Obstbau. Etwa 7 Mühlen befinden sich zersträut inmitten der Dorfhäuser. Je zwei Mühlsteine versorgen örtliche Kunden mit kleinen Mengen von Mehl.

Einige sind kleine Bauten aus Lehmziegeln, andere sind im Souterain normaler Dorfbauten aus Stein und Holz untergebracht.

Prizreni: In dieser alten Stadt, ein historisches Denkmal, befinden sich nur drei Mühlen, obzwar hier früher mehr als 20 Mühlen tätig waren. Zwei Mühlen mit je 2 und 3 Steinen befinden sich in der Nähe von Prizren in Marash. Die dritte Mühle ist die grösste ihrer Art in Kosova, mit 5 Steinen und mit einer Jahresproduktion von 130 000 kg Mehl. Sie sind gebaut aus Lehmziegeln, Lehm und Holz.

Strelci: Mühlen in steinernen Bauten, mit höchstens zwei Steinen im Souterain der Häuser. Sie stehen in engen Tälern under der Gebirgskette des Massivs Bjeshket e Nemuna (Prokletije). Die Tagesproduktion ist zimlich gering, bis 500 kg Mehl pro Tag, je nach dem Wasserstand.

Kabashi: Hier liegen etwa 4 Mühlen an reichlichen Wasserläufen am Abhang der Mokra gora (Bjeshket e Mokres), sie mahlen das ganze Jahr hindurch und bedienen eine breitere Umgebung.

Hulaj: Eine Mühle aus Stein und Holz gebaut, mit zwei Steinen. Sie vorsorgt die wenigen Landwirte in der Umgebung wie auch die Hirten auf den Weiden. Hier waren interessante Schädlinge vorhanden mit allerlei Krankheiten.

Gefundene Insekten

Die in den Mühlen gesammelten Insekten vertreten drei Hauptgruppen: die Vorratschädlinge, ihre Prädatoren und Verzehrern ihrer Überreste und die Parasiten beider. Daneben fanden wir noch Insekten, die in den hölzernen Konstruktionen alter Häuser leben, doch mit Getreide oder Mühlwaren nichts zu tun haben. Zur ersten Gruppe gehörten *Ephestia kühniella* Zell., *Tribolium castaneum* Hbst., *T. confusum* Duv., *Tenebrio molitor* L., *Tenebrionides mauretanicus* L., *Calandra granaria* L., *C. oryzae* L., und *Rhizopertha dominica* F. In die zweite Gruppe reihen wir *Attagaenus pellio* L., *A. piceus* Oliv., *Att. fasciolatus* L., *Dermester lardarius* L., *Lemophlaeus ferrugineus* (Steph), *Cryptophagus sp.* und *Gracilia albanica* Csiki. In der dritten Gruppe befinden sich *Rhyssa vesicatoria*, verschiedene *Ichneumoniden*-Arten, *Tachina sp.*, *Habrobracon hebetor* u. a. Von den Holzschädlingen trafen wir *Anobium punctatum* und *Anobium sp.*

Tabelle 1 zeigt die Verteilung der Funde in einzelnen Mühlen des studierten Gebietes. Die Resultate der Sammlung entsprechen der direkten Sammelmetode, die benutzt wurde. Insekten in Versteckten und Lücken oder solche, die gerade in unbeweglichen Stadien vorlagen (Eier, Puppen), wurden meistens nicht gefangen. Deswegen fehlt in der gesammelten Serie eine Menge weit verberiteter Schädlinge wie *Ephestia elutella*, *Plodia interpunctella*, *Sitotroga cerealella*, *Sitodrepa panicea* und viele andere. Dagegen kommen lokale Elemente vor, wie *Calandra oryzae* oder *Gracilia albanica*. Quantitative Beziehungen sind höchstwahrscheinlich nicht sehr wichtig, da sie nach der Frequenz der Arbeit in der Mühle schwanken. Trotzdem ist es interessant, dass die Gruppe der Prädatoren so reich ist. Ihre Populationen scheinen viel beständiger sein als die schnell auflammende und wieder verschwindende Gruppe der Vorratschädlinge. Bei den Untersuchungen der

Tabelle 1

Übersicht der Insektenfauna der untersuchten Mühlen des Kosova Gebietes

	Mühlen										
	Shtimla	Radavci	Carabregu	Dubova	Prizreni	Isniqi	Tekul	Strelci	Kashica	Kabashi	Hulaj
Schädlinge											
Ephestia kühniella	29	115	34	7	7	51	114	38	6	10	10
Tribolium confusum T. castaneum	—	200	16	5	—	62	24	9	10	—	31
Tenebrio molitor	—	35	7	5	10	8	2	2	—	4	10
Tenerbrionides mauretanicus	—	2	2	—	1	2	2	1	1	1	4
Calandra granaria	—	16	1	—	—	—	—	—	—	—	1
Calandra oryzae	—	13	3	—	—	—	—	—	—	—	3
Rhizopertha dominica	—	—	—	—	—	—	—	—	—	3	6
Prädatoren											
Attagaenus fasciolatus	2	10	1	—	—	—	—	2	3	—	6
A. piceus	—	—	—	—	1	3	1	—	—	—	1
A. pellio	3	—	—	—	6	4	3	—	—	—	5
Dermestes lardarius	—	2	—	—	—	—	—	—	—	—	6
Lemophlaeus ferrugineus	—	—	—	—	—	4	—	6	11	—	—
Cryptophagus sp.	—	—	—	—	—	2	—	1	—	—	—
Gracilia albanica	—	—	—	10	—	—	—	3	3	—	16
Parasiten											
Ichneumonidae div. sp.	—	3	11	6	—	7	—	6	6	11	23
Rhyssa persuasoria	—	2	6	—	—	—	—	3	3	7	8
Habrobracon hebetor	—	3	2	—	—	—	—	4	6	—	2
Tachina sp.	—	6	—	—	—	—	—	1	—	3	3

Prädatoren auf eventuell vorkommende Erkrankungen wurde festgestellt, dass sie eine sehr wichtige Rolle spielen bei der Übertragung der Krankheiten. Im Darm von Attagaenus oder Dermestes fanden wir viele Sporen der Protozoen, die normalerweise in den Vorratschädlingen vorkommen und mit ihrer Nahrung verzehrt werden und den Darm ohne grosse Beschädigung durchlaufen. Doch viel interessanter war in dieser Hinsicht *Tenebrio molitor*. In seinen Larven waren im Darm riesige Mengen von Sporen von *Farinocystis tribolii*, in anderen Fällen eine Mischung von *Nosema withei* und *Thelohania ephestiae* zu finden, so dass auf einfachen Ausstrichen das ganze den Eindruck hervorrief, dass die Larven infiziert sind. Doch konnten keine Gewebe mit Infektion aufgefunden werden und auch waren viele Sporen von *Farinocystis tribolii* im Darmlumen von *Tenebrio* an beiden Polen geöffnet und leer. Doch sind die Sporen im Kot der Larven und Käfer infektiös und *Tenebrio* dient meistens als wichtiger Faktor bei der Verbreitung der Krankheiten, deren Sporen aus toten Insekten nur schwierig befreit werden

können. Eigentlich ist dies nur auf zweierlei Weisen, möglich durch Zermahlen des Getreides und durch die Tätigkeit der Prädatoren.

Nachgewiesene Krankheiten

Trotz der niedrigen Anzahl der vorhandenen Schädlinge und trotz kurzfristiger Lagerung der Mühlwaren in den studierten Mühlen, war der Befall der Schädlinge durch Krankheiten ziemlich hoch. Tabelle 2 gibt eine Übersicht der festgestellten Krankheiten bei 5 Schädlingen: *Ephestia kühniella*, *Tribolium castaneum*, *Tr. confusum*, *Tenebrionides mauretanicus* und *Tenebrio molitor*. Der Befall durch Eugregarinen, wie *Gregarina polimorpha*, *Gregarina steini*, *Gregarina cuneata* ist angegeben doch riefen diese Protozoen keine nachweisbare Schädigung der Tiere, darum sie sind als blose Darmpassanten zu betrachten. Alle Übrigen Krankheiten haben einen sehr entscheidenden Einfluss auf die Vorratsschädlinge und bedeuten wichtige biologische Faktoren der Bekämpfung.

Mattesia dispora Naville ist eine Schizogregarine mit Schiffchenartigen Sporen (Abb. 1) von $11-14\times6{,}5-7{,}5$ μm Grösse. Diese öffnen sich bei der verdickten Pole und wurmartige Sporozoiten werden frei, die das in Gewebe hineinkriechen und dort durch reichliche schizogoniale Teilungen grosse Mengen von Trophozoiten, Gameten und zuletzt Cysten bilden, in denen wider Sporen ent-

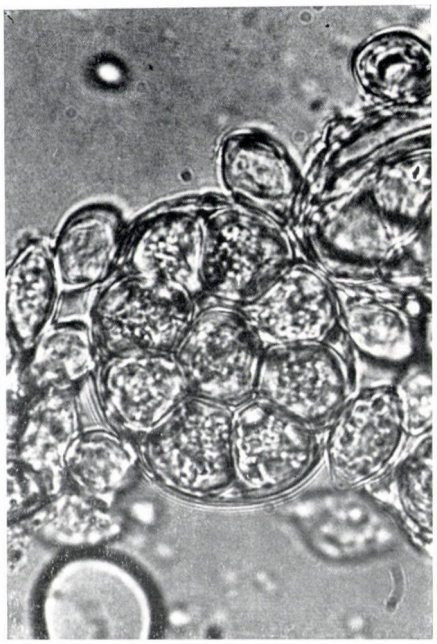

Abb.1. *Adelina mesnili* in *Ephestia kühniella*, eine reife Sporocyste, ringsherum sind die reife Sporen von Mattesia dispora, Mischinfektion, nativ 1000×. Institut für Cytopathologie, Ales, Frankreich

stehen. Die Erkrankung befällt den Fettkörper der Larvan Puppen und Imgaines von *Ephestia Kühniella*. Von diesem Wirte können sie auch auf andere Lepidopteren, wie *Achroa grisea*, *Plodia interpunctella* oder *Galleria mellonella* übergehen und auch dort ist die Erkrankung tödlich. Befallene Raupen sind zuerst Milchweiss, schwer beweglich und verlassen meistens das Netz, das sie im Mehl oder auf Gegenständen weben. Zuletzt ist die Raupe verkürzt abgemagert und rosa gefärbt. Die Erkrankung greift besonders reiche Populationen an und bringt die Gradation in kurzer Zeit zu Ende. Die Verwendung dieser Erkrankung zur biologischen bekämpfung ist zimlich leicht, es genüt infizierte Räupchen in eine gesunde Population hineinzutragen. WEISER (1954, 1956) beschreibt Einzelheiten der Erkrankung.

Farinocystis tribolii Weiser 1954, eine andere Schizogregarine ist typisch für *Tribolium castaneum*. In anderen Tribolium-Arten ist es selten und wird dort durch *Adelina* und *Nosema* ersetzt. Die Schiffchenförmigen Sporen unterscheiden sich von der vorherigen dadurch, dass an beiden Polen rundliche Stöpsel herausragen und diese werden losgelöst wenn die die Sporen in den Darm eines empfindlichen Wirtes kommen. Im Fettkörper der befallenen Larven und Käfer häufen sich Sporenmassen an, und füllen die Zellen. Kranke Larven verenden meist im letzten Instar. Wenn alte Larven oder Käfer mit dem Mehl Sporen in den Darm

Tabelle 2

Überblick über die Verteilung der Pathogene bei einigen Schädlingen der Mühlwaren aus Samlungen in Mühlen von Kosova

	Shtimla	Radavci	Carabregu	Dubova	Prizreni	Isniqi	Tekuli	Stelci	Kashica	Kabashi	Hulaj
Ephstia kühniella											
Gregarina polimorpha	—	—	—	11	—	—	2	—	1	1	—
Mattesia dispora	7	35	4	—	3	5	17	8	—	6	4
Nosema heterosporum	—	—	11	—	7	5	—	8	—	2	3
Thelochania ephestiae	—	10	—	4	8	18	10	—	—	—	—
Bacillus thuringiensis	—	—	5	—	7	8	8	18	22	9	21
Tribolium castaneum tribolium confusum											
Adelina tribolii	—	18	—	—	9	—	11	—	8	5	13
Farinocystis tribolii	—	—	—	—	—	3	7	—	6	—	6
Nosema whitei	—	—	—	7	7	—	5	—	11	—	—
Tenebrionides mauretanicus											
Farinocystis tenebrionides	—	—	—	—	—	11	16	—	5	—	3
Nosema tenebrionides	—	—	—	—	—	5	6	—	4	—	—
Tenebrio molitor											
Gregarina steini	—	6	8	—	4	—	—	2	—	—	8
Gregarina cuneata											

bekommen, verlassen Sporozoiten die Sporen und die Infektion entwickelt sich im Käfer. Deswegen finden wir an einem bestimmten Ort, bzw. zu einer bestimmten Zeit entweder nur Larven, oder Imagines infiziert. Die Erkrankung wird durch Kannibalismus der Larven, durch befressen der Kadaver soeben verstorbener infizierter Tiere übertragen. Das Ausfressen toter Tiere ist umso häufiger, je weniger Proteine anderen Ursprungs in der Nahrung vorkommen. Dagegen bei Zugabe z. B. trockener Heffe, Soya, oder Erbsenmehl usw. Kommt die Infektion zum Stillstand und die Population wird allmählich asaniert.

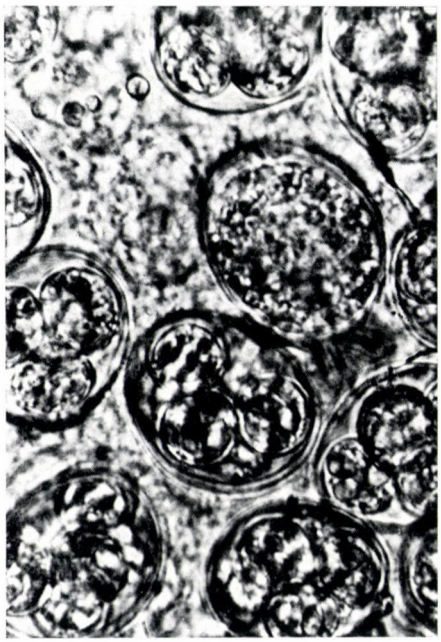

Abb. 2. *Adelina tribolii* in *Attagaenus pellio*, Gameten, unreife und reife Sporocysten, nativ 1000×, Institut für angewandte Zoologie, der Max. Universität, München BRD

Adelina tribolii Bhatia ist eine Coccidie mit rundlichen Sporen (Abb. 2) die dauerhaft in einer Zyste unter der Cystenwand angehäuft liegen. Von BHATIA (1937) wurde sie beschrieben und ihr Auftreten in Tribolium studiert. Sie kommt in allen Tribolium – Arten vor und verursacht hohe Mortalität in den Altlarven. Sie geht nicht auf andere Käfer über, doch kann sie im Darm auch bei Arten vorkommen, die sie nur mit der Nahrung bekamen. Dieser Fall wurde auch in unserem Materiale bestätigt.

Neben den schon beschriebenen Sporozoen möchten wir noch die bei *Tenebrionides mauretanicus* aufgefundene *Farinocystis* näher besprechen. Die Erkrankung befällt hier auch den Fettkörper und die Fettzellen werden durch Massen von Sporen ausgefüllt. Die Masse der Sporen, die aus dem Fettkörper

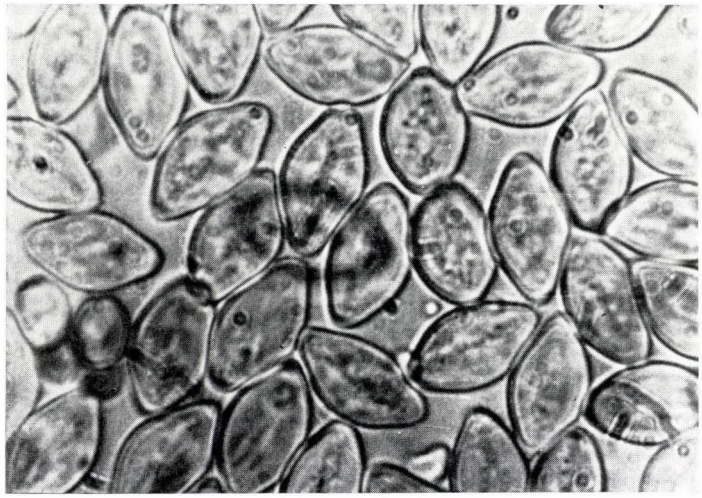

Abb. 3. *Farinocystis tenebrionides sp. n.* in *Tenebrionides mauretanicus*, freie und reife Sporen, nativ 1000×. Landwirtschaftliche Fakultät, Prishtina, Jugoslawien

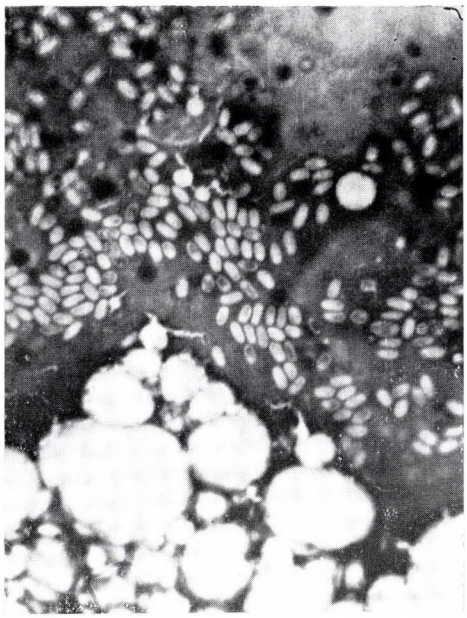

Abb. 4. *Nosema tenebrionides sp. n.* in *Tenebrionides mauretanicus* reife Sporen, Giemsa — Färbung, 1000×, Landwirtschaftliche Fakultät, Prishtina, Jugoslawien

befallener Larven herausfliesst besteht aus jungen, einkernigen Sporen, Sporen mit fortschreitender Kernteilung bis zu 8-kernigen Stadien und reifende und reife Sporen mit ausgebildeten wurmartigen Sporozoiten. Die Sporen sind navikular $6-7 \times 13$ μm gross, dickwandig (Abb. 3). Sie unterscheiden sich besonders durch die dickere Wand 0,5 – 0,7 μm) von *F. tribolii*. Die meisten Sporen liegen einzeln, doch finden wir auch Gruppen von 16 bis 32 zusammen.

Die Frage, ob es sich um eine Art mit Wirt-bedingten Variationen handelt oder um zwei Arten handelt, wird das Studium der Ultrastrukturen entscheiden.

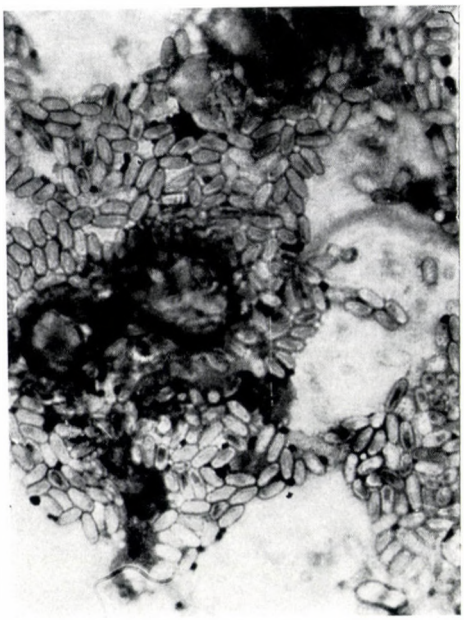

Abb. 5. *Nosema whitei* in *Tribolium castaneum*, reife Sporen, Giemsa-Färbung, 1000×. Landwirtschaftliche Fakultät, Prishtina, Jugoslawien

Bisweilen halten wir den Parasiten aus *Tenebrionides mauretanicus* aus den Mühlen zu Isniqi, Tekuli, Kashica und Hulaj für einen Stamm der Farinocystis tribolii.

Über die neue Nosema-Art, *Nosema tenebrionides sp. n.* (Abb. 4) in *Tenebrionides mauretanicus* L. sowie über *Bacillus thuringiensis* und andere neue Arten der Pathogenen wird zu späterer Zeit berichtet werden.

Zu den Mikrosporidien gehört die in *Tribolium castaneum* und *T. confusum* von WEISER (1953) beschriebene *Nosema withei* (Abb. 5). Die Sporen messen $4,5-5 \times 2-2,5$ μm, sind aber birnförmig, an beiden Enden breit abgerundet. Sie begleiten die Infektionen mit *Farinocystis* und *Adelina*.

Die Mikrosporidie aus *Ephestia kühniella* entspricht der von MATTES (1928) aus *Ephestia kühniella* beschriebenen *Thelohania ephestiae*. WEISER (1953, 1954) hatte diesen Krankheitserreger nicht in seinem Material, doch hier ist sie sehr

häufig. Wie schon bei MATTES beschrieben, kommt diese Art in zwei Formen vor, in achtgliedrigen Pansporoblasten, die der *T. ephestiae* gut entsprechen und dann noch zumeisst als einzelne Sporen. Diese Anomalie wurde auch bei anderen Mikrosporidien beobachtet, wie z. B. bei *Nosema necatrix (Thelohania diazoma)*, die darum von KRAMER (1965) als zwei verschiedene Arten beschrieben wurde. Die Angaben bei MATTES, der die Sporen auf fixiertem Schnittmaterial gemessen hat, sollen noch mit Massen frischer Sporen ergänzt werden. Einzelne Sporen messen 2,5 ± 0,3 × 4,0 ± 0,5 μm, Sporen in Achtern messen 2,3 ± 0,3 × 3,5 ± 0,5 μm und sind leicht nierenförmig gekrümmt, im Gegenteil zu den regelmässig ellyptischen einzelnen Sporen. Die Mikrosporidie befällt den Fettkörper, die Oenocyten und später den Hypoderm der Rauppen und verursacht hohe Mortalität. Die achtsporigen Formen kommen unter einzelnen Sporen unregelmässig vor, in einigen Tieren sind achtgliedrigen Pansporoblaste überall verbreitet. In anderen, besonders in jungen Infektionen, sind Pansporoblasten selten.

Die als *Nosema heterosporum* bezeichnete Infektion ist durch lange, ovale Sporen gekennzeichnet, 4 × 2,5 μm. Achtsporige Gruppen fehlen völlig.

Die *Bacillus thuringiensis* Infektionen wurden einerseits auf Grund toter Larven mit Sporen von *Bacillus thuringiensis* festgestellt, anderseits kamen auch Larven vor, die nach dem Transport in das Labor eingingen und *B. thuringiensis* Sporen aufwiesen. Die befallenen Tiere wurden grau, später schwarz gefärbt und enthielten Massen von Sporen die Stämme wurden isoliert und sind weiter erwertet.

Diskussion

Die in beiden Tabellen angeführten Fälle von Mühlen in Kosova und die in ihnen vorkommenden Schädlinge, Prädatoren und Parasiten, wie auch Krankheiten geben ein Bild über die Verbreitung dieser Faktoren unter natürlichen Bedingungen, ohne Eingriffe durch Insektiziden. Es zeigte sich, dass ohne periodischen Einsatz in chemische Mitteln die biologischen Mittel immer bis zu einem bestimmten Grade vorhanden sind und sich im Abhängigkeit von der Populationsdichte ihrer Wirte mehr oder weniger verbreiten. In der Verbreitung einzelner Schädlinge und Krankheiten konnten wir in dieser Serie keine besondere Unterschiede feststellen, doch gibt es andere Fälle aus mehr entfernten Lokalitäten, die noch der Bearbeitung harren, Beispiele von völlig lokal verbreiteten Infektionsherden, Weitere Unterschiede werden auch andere Sammelmethoden bringen, besonders die Anwendung von Fallen und die Zuchtmetode wo gesammeltes Material in weiteren Generationen durchgezüchtet wird und bei Anhäufung auch die verborgenen Infektionen zum Vorschein bringt.

Danksagung

Für die grosse Hilfe bei der der Bestimmung und Bearbeitung der Pathogene-Arten, sowie auch bei der Anlage des Manuskriptes danke ich Herrn Dr. J. WEISER Dr. Sc. Entomologisches Institut der Akademie Prag, Tschechoslowakei.

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Temporary Inhibition of Diapause Incidence by Juvenoids in the Cereal Bug, *Eurygaster maura* L. *(Heteroptera: Scutelleridae)*

By

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Adults of *Eurygaster maura*, collected in wheat fields, were topically treated with 50 and 200 μg dosages of juvenoids R-20 458 and HZ-33, respectively. The juvenoids interfered with the incidence of obligatory imaginal diapause by prolonging the summer feeding period and by inducing premature reproduction. In proportion to the doses applied, deposition of mostly fertile eggs was observed. The majority of eggs, however, could not complete embryogenesis due to a possible indirect ovicidal effect of the chemicals. The perspectives of field use of juvenoids against cereal bugs is discussed.

In connection with the fluctuations of their population levels, diverse opinions have been expressed on the economic significance of cereal bugs in Hungary. It is a fact, however, that if the weather conditions favour the build-up of a gradation, considerable crop losses can be predicted. So the importance of control measures against these pests, always present in the cereal crops, can not be neglected. In our country *Eurygaster maura* L. and *Eurygaster austriaca* Schrk. are the most dangerous species, especially for wheat. Their life-cycles as well as their damage resemble to those of *E. integriceps*, a serious pest in Eastern-Europe and Balkan.

Similarly to the other cereal bugs, *E. maura* has an obligatory imaginal diapause. After a short period of intensive feeding in the late summer (prediapause) adults migrate to hibernation sites (these are in Hungary mostly forests on slopes of hills and mountains) and overwinter in the litter. In the spring the bugs become active and invade the grain fields again.

The artificial breaking of reproductive diapause with chemicals, e.g. with the use of juvenile hormone analogues was suggested as a perspective control method against some insect pests. A forced activity under unappropriate ecological conditions was assumed to decimate or eradicate the overwintering adult population or its possible progeny. Topical treatments with juvenile hormone or its analogues successfully terminated imaginal diapause in various *Coleoptera* such as *Hypera postica* (BOWERS and BLICKENSTAFF, 1966), *Oulema melanoplus* (CONNIN *et al.*, 1967), *Leptinotarsa decemlineata* (DEWILDE, 1969; SCHOONEVELD, 1973; DEWILDE and LUTKE SCHIPHOLT, 1974), and ladybirds (HODEK *et al.*, 1973;

ALI *et al.*, 1974a). Similar effect was observed on a leafhopper (KAMM and SWENSON, 1972). Juvenoid applications reactivated also hibernating adults of *Eurygaster integriceps* (BUROV *et al.*, 1972; KONTEV *et al.*, 1974). They induced prolonged feeding and sexual activity in the laboratory. In the field, however, sprayings with JH analogues failed to produce satisfactory effectivity on adults of this species (KONTEV *et al.*, 1974).

In our investigations we wanted to study the capacity of juvenoids for inhibition of diapause incidence by treating adults of *Eurygaster maura* in the pre-diapause stage. The same approach to interfere with insect diapause was reported concerning a Coccinellid (ALI *et al.*, 1974b).

Material and Methods

In July and August young adults of *Eurygaster maura* were collected on wheat fields near Mosonmagyaróvár (West-Hungary). The bugs were found still feeding, i.e. in a state before migration. According to the rearing method described by RÁCZ (1975), the insects were placed into glass jars of cca. 500 ml volume and supplied with drinking water as well as seeds and young seedlings of wheat. After treatments, the rearings were kept in the laboratory at 22–25°C and at natural late summer-autumn photoperiod.

For topical applications two juvenoids were used: 1-(4'-ethyl-phenoxy)-6,7-epoxy-3,7-dimethyl-2-octene (R-20 458; Stauffer Chem. Comp.) and 1-(4'-chlorophenoxy)-6,7-epoxy-3,7-dimethyl-2-octene (HZ-33; Institute of Organic Chemistry and Biochemistry, ČSAV). The 50 and 200 μg dosages, resp., were administered in 2 μl acetone to the last tergites of abdomen covered by the wings. Control insects received only acetone. In each experimental group 6 males and 6 females were treated. After treatments, observations were made in daily intervals regarding the period of oviposition, the number and hatchability of eggs as well as the mortality in both sexes.

Results

The results of two separate experiments are summarized in Table 1. In one trial the compound R-20 458 was applied, while in the other one, performed 20 days later, we used the juvenoid HZ-33. The corresponding curves demonstrating the cumulative egg numbers, are presented in Figs 1 and 2.

In both experiments, the juvenoids prolonged the feeding period of adults and induced reproduction for a certain time interval. On the contrary, in control groups no signs of reproductive activity was observed. In treated groups, matings were seen only in a few cases but the females laid mostly fertile eggs showing that insemination actually occurred.

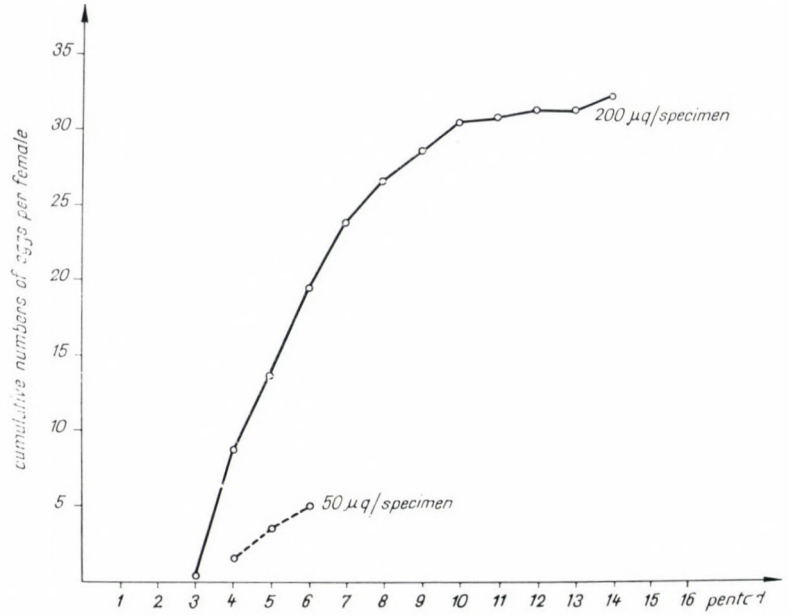

Fig. 1. Oviposition of *Eurygaster maura* females treated with the juvenoid R-20 458. (The egg numbers were calculated for each decade)

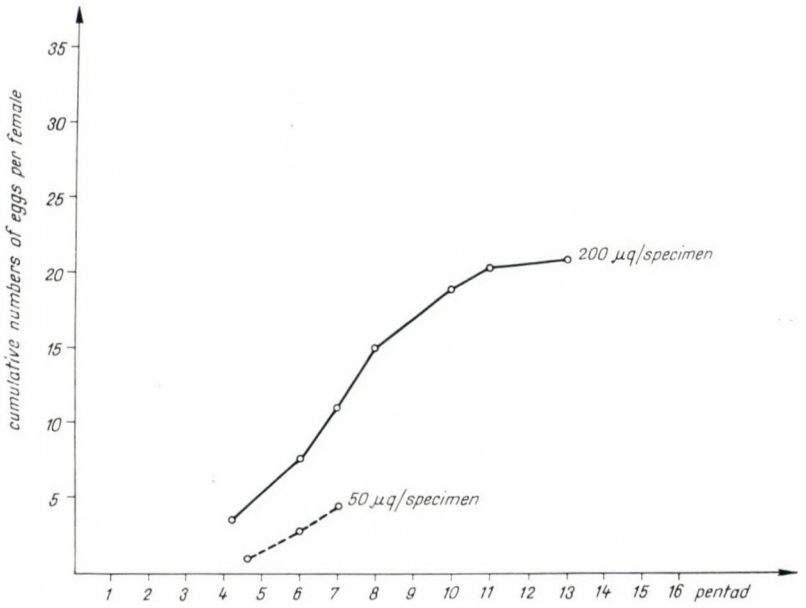

Fig. 2. Oviposition of *Eurygaster maura* females treated with the juvenoid HZ-33. (The egg numbers were calculated for each decade)

Table 1

Effect of juvenoids, applied in the pre-diapause stage, on the reproduction and survival of *Eurygaster maura* adults

Date of treatment	Compound	Dosage µg/spec.	Total No. of eggs	Embryonal development (%)			Mortality (%)	
				sterile eggs	died embryos	hatched larvae	males	females
Aug. 8	Control	0	0	—	—	—	0	0
		0	0	—	—	—	10	0
	R-20458	50	41	12.2	87.8	0.0	0	8
		50	15	6.7	93.3	0.0	8	0
		200	222	17.6	82 4	0.0	8	0
		200	168	11.6	88.4	0.0	17	17
Aug. 28	Control	0	0	—	—	—	8	0
	HZ-33	50	22	9.1	90.9	0 0	8	8
		200	126	0.8	82 5	16.7	8	0

After applying 50 µg of R-20 458, the oviposition started within 17 to 21 days and proceeded for 5 to 9 days. The 200 µg dosage of the same compound provoked egg-laying already on the 13th or 17th day after treatment. This reproductive activity lasted then for 36 to 51 days. The other juvenoid, HZ-33 in 50 and 200 µg dosages induced oviposition within 23 and 21 days, resp., and freshly laid eggs were found thereafter for 38 and 40 days, respectively. In both trials, the total numbers of eggs can be correlated with the doses applied (Table 1).

Figures 1 and 2 also demonstrate that the activation of reproductive processes by low doses was rather weak and transitional. On the contrary, after using high dosages, the hormonal induction seemed much more expressed and stable. Most eggs were deposited in the first half of the active period.

Attention was also paid to the development of eggs laid by treated females. As Table 1 shows, the fertility of eggs does not seem to be affected since the percentages of sterile eggs did not exceed 17%. The chemicals acted first of all to the hatchability of eggs. Generally 100% of the fertile eggs revealed a developmental block independently of the doses applied. Before death, the embryonal development proceeded until the formation of nearly perfect larva.

The mortality rates during the 2 months period of investigation remained fairly low (Table 1). Even the high dosages did not appear toxic for the insects.

Discussion

Eurygaster maura, one of the most important cereal bugs in Hungary, can be readily reared in the laboratory (RÁCZ, 1975). Low mortality of adults was also found in our trials. The bugs, brought in from wheat fields in the late summer exhibited still feeding activity. Since the subsequent migration in the nature can

be considered as entry into diapause, the developmental stage of our experimental insects could be denoted as pre-diapause.

The juvenoids used, R-20 458 and HZ-33 proved effective also on *E. integriceps* (KONTEV *et al.*, 1973; KONTEV *et al.*, 1974; BUROV *et al.*, 1976b). In our experiments they could induce reproduction in a relatively early phase of imaginal life when the chemical disturbation of diapause in *E. integriceps* seemed still problematic (BUROV *et al.*, 1972). The 50 μg dosages produced merely a very slight and temporary reproductional activity which became evident by oviposition only after 17 to 23 days. We assume that in these cases only a few females deposited eggs. After treatments with 200 μg doses, the egg-laying started a little earlier and lasted for much longer periods. In the deposition of a considerable number of eggs supposedly all of the females participated. Similar relationship between dosages of juvenoids and some quantitative data on reproduction was reported for *E. integriceps* (BUROV *et al.*, 1972; KONTEV *et al.*, 1974). We could not find any difference between the activities of the two juvenoids administered to *E. maura.*

We have to emphasis that in each experimental group the inhibition of diapause incidence by juvenoids proved only temporary and after finishing the food-uptake and reproduction the bugs entered the diapause stage. Nevertheless, they are supposedly not able "to forget" this extra active period of their life. It can be supposed that by exposing them again to natural environmental conditions, with the depleted food-reserves they would have had much lower chance for a successful overwintering.

The considerable reduction of developmental capacity of eggs can be less easily explained. Our data equivocally show that the fertilisation of eggs was not affected. Taking into consideration the results of BUROV *et al.* (1976a) on *E. integriceps*, at the time of our treatments or when the first mating occured the males were already sexually mature and competent for producing normal sperm. Since the developmental block was generally found near the end of embryogenesis, we may assume an indirect ovicidal effect of juvenoids through the reproductive organs of females. As BUROV and SHEKHTMAN (1976) described, young larvae of *E. integriceps* often die within the egg-shells if low dosages of JH analogues were administered to females or if the time interval between juvenoid treatment and oviposition was long.

The field use of juvenoids against cereal bugs can be perspective if the activity and environmental stability of the compounds are high and if the direct contact of sensitive developmental stages can be ensured (KONTEV *et al.*, 1973; KONTEV *et al.*, 1974; BUROV *et al.*, 1976b). At present the action on the larval-imaginal transformation or on the imaginal diapause seem to be the best possibilities. In both cases we have to face huge technical difficulties as well.

By treating adults still in their active life preceding the diapause, the direct contamination of insects enables a more perfect hormonal action than if one tryes to spray the environment of hibernation sites. The forest where *Eurygaster* imagos overwinter in Middle-Europe, are too large and generally unaccessable for spray-

ings. The application of juvenoids on grain fields before mass-migration of bugs may postpone the incidence of diapause. This way the vitality as well as the capacity of insects for migration and hibernation would be considerably reduced. Even if they produced some viable offspring in the autumn, it would not have any practical importance, as the latter could not finish their development before the winter frost due to the lack of appropriate food and to the unfavourable weather conditions. Also a combined application of juvenoids could be suggested against a mixed population of larvae and adults when partly the metamorphosis, partly the imaginal diapause would be affected.

Treatments in the pre-diapause period need higher dosages than at the time of diapause. We hope, however, that by using more active and stable juvenoids in the future, this obstacle can be overcome.

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Study on the Factors Influencing the Decomposition of Linuron.

I. The temperature and pH dependence of the linuron decomposition

By

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By studying the decomposition of linuron at temperatures 10—80°C and at pH values 4—10, the activation energy of decomposition was found 21.8 kcal/mol, with a standard deviation of 2.2 kcal/mol. It has been established that the activation energy does not depend considerably on the pH value of the system. The decomposition shows in diluted aqueous solutions the characteristics of a reaction of first order, the decomposition product is 3,4-dichloro-aniline. The rate constants of the reaction are higher at pH values far from neutral (pH 4 and 10) than at values near to the neutralization point (pH 6 and 8). The rate constants measured at different pH exhibit a nearly identical temperature dependence, the average Q_{10} value is 2.77.

For herbicides a very important requirement is the selective action, as well as the inactivity against the beneficial soil microorganisms. It is also important both from point of view of environmental protection and agriculture that — after having exerted its effect — the herbicide be decomposed to a biologically inactive compound and should not accumulate in the soil.

The tri-substituted carbamide derivatives comply with the requirements mentioned above; the one of the most common representants of this group is linuron, with the formula N-(3,4-dichlorophenyl)-N'-methoxy-N'-methylcarbamide (WALKER and SCHMIDT, 1974; TATSUMAYA and JIKIHARA, 1970; VENKATARAMAN and RAJYKALAKSHAMI, 1971).

The decomposition of linuron has been followed by colorimetric methods (FRIESTAD, 1974) and by ECD gas chromatography (MCKONE, 1969). Linuron was extracted from the soil by using aqueous acetone (STONOV and FOFANOV, 1972) or by hexane (USOROH and HANCE, 1974).

No uniform opinion was formed in the literature as regards both the rate of decomposition and the factors which influence the process. This resulted from the differences in the soils and the soil microflora studied on one hand and on the lack of separation of the factors which play a role in the decomposition on the other. Some authors (WALKER, 1973; MOYER *et al.*, 1972) regard the soil adsorption as an important factor, others are of the opposite opinion (HANCE, 1974; GEISSBÜHLER *et al.*, 1963); the organic soil components increase the linuron decomposition according to some authors (SAVAGE, 1973), others deny it (HANCE, 1973).

Regarding the rate of decomposition, the data of the different authors are hardly comparable, as the rate constants are usually not published. According

to SAVAGE (1972) linuron decomposes within 14 days, BARTHA and PRAMER (1969) did not notice changes for 40 days, GEISSBÜHLER and GUTH (1970) measured an 80% decomposition in 5 months, SAMOSVAT (1970) observed in 4 months a 93–95% decomposition, MAJUMDAR (1969) also in 4 months a 79–94% decomposition. According to STECKO (1972) linuron completely decomposed in one year, while according to MORRIS and PENNEY (1971) linuron may persist in the soil for even more than a year. Some authors regarded the decomposition as a microbiological process and studied the possible role of microorganisms in it: *Bacillus sphaericus* (ENGELHARDT *et al.*, 1971; WALLNÖFER, 1969), *Rhizoctonia solani* (WEINBERGER and BOLLAG, 1972), *Arthrobacter globiformis* (KOSINKIEWICZ, 1973).

In course of the microbiological deactivation first demethylation, then demethoxylation occurs (ROSS and TWEEDY, 1973; WALLNÖFER *et al.*, 1973). The 3,4-dichloro-aniline, formed at the hydrolysis of the carbamide bond, does not accumulate in the soil, but undergoes further decomposition (BÖRNER, 1967).

By studying the literature it became obvious that in most cases only one or some factors were selected and investigated from the entire system of elements, like the physical factors (adsorptive capacity, temperature), chemical factors (pH, organic matter content) and microbial factors (density and composition of the microflora), which renders naturally impossible to make a quantitative evaluation on the interconnections of the individual factors.

By determining the chemical parameters of the decomposition (temperature and pH dependence) and by comparing the rates of decomposition in sterile soils as well in ones with natural microflora, we aimed to get answers on the following questions:

1. How is the linuron decomposition affected by temperature and pH?
2. How influence the physical and chemical characteristics of the soil the linuron decomposition?
3. How can the chemical and microbiological decompositions be separated in soils with different microbial populations?

In present paper our results referring to the pH and temperature dependence of the chemical decomposition are dealt with.

Material and Methods

To study the dependence of linuron decomposition on temperature and pH, a stock solution (20 μg/ml) was prepared. To promote the dissolution of linuron, the latter was solved in 2% ethanol (calculated to the end volume), then diluted with distilled water. To set the pH values, buffer solutions were prepared (pH 4, 6, 8, 10) from the Britton–Robinson buffer series, which can be used over a wide range of pH values (BREZINA and ZUMAN, 1956). In test tubes 6 ml buffer and 6 ml linuron solution were measured, then kept in thermostates at temperatures of 10, 20, 40, 60 and 80°C; the exposure times were in case of 80°C 20 hours, for 60°C 8, for 40°C 14, for 20 and 10°C 80 days. Each decomposition curve was

determined with 10 – 10 measuring points. In given time periods samples of 1 ml volume were taken (cooled if it was necessary), extracted with 1 ml hexane and the hexane solution was analyzed by ECD gas chromatography. By plotting the logarithms of the measured concentrations as functions of incubation periods, a linear regression was calculated. The slope of the curve was equal to the rate constant of the reaction. From the rate constants measured at different temperatures but at the same pH value, the activation energy of the decomposition was calculated, based on the ARRHENIUS formula:

$$\Delta H_a = \log \frac{k_2}{k_1} \frac{2.3 \cdot R \cdot T_1 \cdot T_2}{T_2 - T_1}$$

where k_1 and k_2 are the rate constants measured at T_1 and T_2 Kelvin temperatures, respectively, R gas constant = $1.987 \cdot 10^{-3}$ kcal/grade.

The activation energies measured at different pH values were compared to establish whether there are significant differences among them, then calculated the average activation energy and the standard deviation. Between logarithms of the rate constants measured at different temperatures but at identical pH values and the incubation temperatures, linear regression analysis was carried out, which indicated the grade of dependence of the decomposition from the temperature and pH.

To identify the hydrolysis product of linuron, we dissolved 5 mg linuron in 1.5 ml ethanol, added 1.5 ml 2 *N* NaOH solution and incubated at 70°C for 5 hours. The solution was then cooled and applied as stripes on 4 Kieselgel HF_{254} (20 × 20 cm) thin-layer chromatography plates. The plates were then developed by a 95 : 5 benzene-acetone mixture, then air dried. The two fractions visible in UV light were scraped off, the identical fractions harvested on the 4 plates were united on a G_3 filter, then washed with 150 ml benzene. By evaporating the solvent, we took the infrared spectra of the residues, of pure linuron and also of the possible decomposition product, 3,4-dichloro-aniline.

Results and Discussion

The regression lines calculated between the logarithms of linuron concentration and the incubation periods showed a significant linear regression, the reaction may be regarded therefore under the circumstances studied, as of first order; this is characteristic for hydrolytic processes occurring in aqueous diluted solutions. The rate constants measured at pH 4 and 10 are significantly higher than the ones got at pH 6 and 8, i.e. nearer to the neutral region. As example for the decomposition rate the results got at 60°C are shown in Fig. 1.

The activation energy data belonging to different pH values are contained in Table 1.

Table 1

Average activation energies of linuron decomposition at different pH values

ΔH_a kcal/mol			
pH 4	pH 6	pH 8	pH 10
19.9	23.8	19.6	21.6

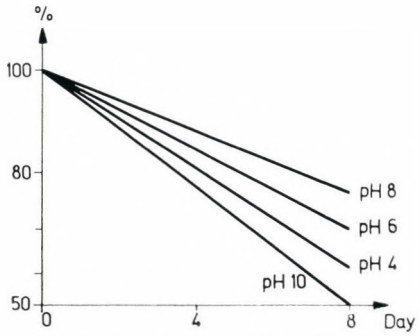

Fig. 1. Linuron decomposition at 60°C

Table 2

Linear regression lines of the temperature dependence of reaction rate constants

pH	Y = bX + a	(Y = log k−2; X = temperature)		
	4	6	8	10
b	0.0412	0.0472	0.0405	0.0428
a	−1.70	−2.32	−2.18	−1.53
s_b	0.0020	0.0026	0.0031	0.0577
r	0.99873	0.99842	0.9806	0.98847

As it can be observed from Table 1, the given standard deviation does not make possible to establish significant differences at the 95% level between the activation energies, therefore the average activation energy can be calculated: this was found 21.8 Kcal/mol, with a standard deviation $s = 2.2$ Kcal/mol.

From the study of the infrared spectrum of the decomposition product of linuron obtained by alkaline hydrolysis in alcohol, it was established that under the circumstances mentioned, only 3,4-dichloro-aniline was produced.

The regression lines calculated between the logarithms of rate constants measured at different temperatures at identical pH as well as the temperatures are shown in Fig. 2; their characteristics are indicated in Table 2.

As it can be observed from Table 2, the temperature dependence of rate

constants is of similar character, which is obvious from the identical mechanism of reaction.

The changes in the reaction rate on each 10 C temperature increase yielded an average value of 2.77, by calculating with the correlation k_T/k_{T-10}.

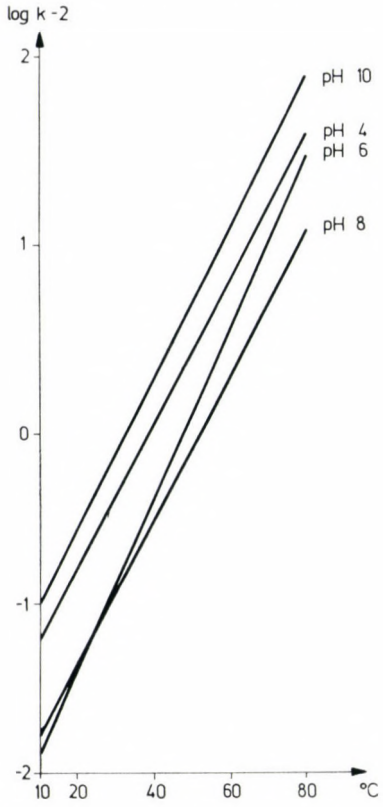

Fig. 2. Temperature dependence of rate constants of linuron decomposition

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The Uptake of Some Benzimidazole Fungicides from Soil into Plants and their Persistence in Soil and Plant Tissue

By

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Soil treatments with benomyl, carbendazim or thiabendazole of potted plants of *Agonis flexuosa* resulted in more rapid uptake by the plants of benomyl and carbendazim than thiabendazole which was apparently adsorbed onto soil particles and taken up more slowly by the plant. This characteristic may make thiabendazole more suitable as a preventative against susceptible foliar pathogens because of this increased retention time.

During work on the control of *Glomerella cingulata* dieback of camellia (BERTUS, 1974) it was found that plants were able to take up and retain in their foliage up to 106 ppm of thiabendazole applied as a soil drench (unpublished results).

The ability to retain significant amounts of fungicide in the foliage could be of considerable value from the point of view of plant protection and the following experiments were designed to study the fates of three benzimidazole fungicides in the plant and soil following application.

Materials and Methods

The test plant selected was *Agonis flexuosa* (Spreng.) Schau. as a number of seedlings of this species was available. Seedlings were grown, ten to a 150 mm diameter pot, in a standard potting mixture of soil, sand and peat. When the seedlings were approximately 15 cm tall the soil in each pot was drenched with 250 ml of an aqueous suspension of fungicide containing 750 ppm of active constituent. The fungicides used were methyl-N-(1-butylcarbamoyl-2-benzimidazole) carbamate (benomyl), methyl-2-benzimidazole carbamate (carbendazim) or 2(4'-thiazolyl) benzimidazole (thiabendazole). Samples of plant tissue and soil were taken for analysis 1 day, 4, 8, 15, 22 and 36 days after treatment. A second lot of plants were given repeat treatments with fungicide 4 weeks and 8 weeks after the first treatment and samples for analysis were taken 4 weeks, 8 weeks and 16 weeks after the last treatment.

Analytical Methods: The methods of analysis were based on the rapid degradation of benomyl in organic solvent solution to methyl-2-benzimidazole carba-

mate (MBC) (CALMON and SAYAG, 1975), conversion of benomyl in organic solvent solution to MBC by evaporating the solvent into 0.1 N HCl on a water bath (PEASE and GARDINER, 1969), and the partitioning characteristics of MBC and thiabendazole (TBZ) with change of pH (WHITE and KILGORE, 1972; Merck, Sharp and Dohme (Aust.) Pty. Ltd.–Personnal Communication).

Samples of soil were collected by taking 20 increments of soil 1 cm below the surface, compositing and sub-sampling; the sub-sample was soxhlet extracted for 8 hours with $CHCl_3$, partitioned into 0.1 N HCl and re-extracted into $CHCl_3$ after adjusting the pH to between 9 and 10 with a borax buffer. Repetition of this partitioning process effectively removed interfering materials co-extracted from the soil. The "cleaned-up" $CHCl_3$ extract was concentrated on a rotatory evaporator where required and extracted in 0.1 N HCl. Absorbance of the fungicides (281 nm for benomyl and MBC, 302 nm for TBZ) was compared to standards of MBC and TBZ in 0.1 N HCl at the same wavelengths. Recoveries of fortified samples at 100 ppm were, benomyl 82 $\pm$ 8%, MBC 88 $\pm$ 5% and TBZ 85 $\pm$ 5%.

Plant tissue samples were obtained by cutting off at ground level a single plant from each treatment. The tissues were homogenized with $CHCl_3$ and 0.1 N HCl (1/1 v/v), centrifuged and the supernatant HCl layer retained for analysis by the method used for soil samples. Recoveries from untreated plants fortified at 50 ppm with the fungicides were benomyl 85 $\pm$ 8%, MBC 93 $\pm$ 5= and TBZ 93 $\pm$ 5%. In the case of benomyl-treated plants the initial centrifuged extract was heated on a water bath to evaporate the $CHCl_3$ (and thus convert any benomyl present to MBC), the remaining acid solution was washed with $CHCl_3$, the $CHCl_3$ layer discarded and the HCl layer analysed as above.

Results and Discussion

The results obtained in the second experiment (Table 2) show that thiabendazole is retained for greater periods in the soil than benomyl or carbendazim under normal nursery watering regimes. This compares with the findings of AHARONSON and KAFKAFI (1975) who found that carbendazim was leached more rapidly from soil columns than thiabendazole. They postulated that the retention of thiabendazole was due to stronger adsorption of this compound onto soil particles and therefore availability for uptake was decreased. Our results also indicate that uptake is restricted compared to benomyl and carbendazim by apparently stronger adsorption characteristics of thiabendazole. However, this adsorption does not render thiabendazole permanently bound to the soil but merely reduces the rate of uptake (Table 1). There was no significant difference in the ultimate levels in the plant attained by all three fungicides.

The apparently decreasing plant fungicide concentrations (Table 2) of benomyl and carbendazim are most likely due to plant growth and represents a dilution of the fungicide rather than a loss from the plant system. An attempt was made to ascertain whether the fungicides were present is new growth occurring

Table 1

Concentrations of fungicides in plant tissue and soil after a single soil drench treatment of *Agonis flexuosa* plants in pots

Time of sampling (No. of days after treatment)	Fungicide	Concentration of fungicide (ppm)	
		Plant tissue	Soil
1 day	Benomyl	<3	22
	Carbendazim	<4	56
	Thiabendazole	<2	32
4 days	Benomyl	<2	20
	Carbendazim	<2	125
	Thiabendazole	<1.5	128
8 days	Benomyl	<7	19
	Carbendazim	31	14
	Thiabendazole	<3	155
15 days	Benomyl	41	10
	Carbendazim	42	10
	Thiabendazole	18	60
22 days	Benomyl	47	5
	Carbendazim	43	6
	Thiabendazole	22	50
36 days	Benomyl	81	<3
	Carbendazim	50	5
	Thiabendazole	24	50

Table 2

Concentrations of fungicides in plant tissue and soil after three soil drench treatments of *Agonis flexuosa* plants in pots

Time of sampling (Weeks after last treatment)	Fungicide	Concentration of fungicide (ppm)	
		Plant tissue	Soil
4 weeks	Benomyl	505	15
	Carbendazim	380	3
	Thiabendazole	320	460
8 weeks	Benomyl	450	3
	Carbendazim	390	5
	Thiabendazole	400	103
16 weeks	Benomyl	480	<1
	Carbendazim	170	<1
	Thiabendazole	440	10

between samplings. Quantitatively this was unsuccessful due to to extremely small sample sizes, however, qualitatively benomyl and carbendazim were not detected in the growing tips two months after the last treatment in the second experiment although a positive indication of thiabendazole residues was observed.

FUCHS, FERNANDES and DE VRIES (1974) state that a reservoir in the planting medium guarantees a continuous supply of fungicide to the plant but no evidence of retention of fungicide was provided. PETERSON and EDGINGTON (1970) showed that the distribution of fungicide within the leaf was dependent on the reservoir in the soil. Our work suggests that the increased retention of thiabendazole in the soil guarantees a reservoir that, on uptake, can be mobilised to the growing tip of the plant. While benomyl and carbendazim may quickly pass into the leaves, the slower release of thiabendazole from the soil would favour a more even distribution of the fungicide within the plant. This means that among the three benzimidazole fungicides tested, thiabendazole would be the most effective prophylactic against foliar infection when applied as soil drenches.

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Book Review

G. M. HOFFMANN, F. NIENHAUS, F. SCHÖNBECK, H. C. WELTZIEN and H. WILBERT: *Lehrbuch der Phytomedizin* (Handbook of Plant Medicine). Paul Parey, Berlin and Hamburg, 1976. 490 p. with 101 Figures. Price: 88 DM

Modern trends both in pest and disease management and in the protection of environment require new type of experts who are called "Phytomediziner" in the German language area and perhaps "plant doctors" in English. This type of expert must be familiar with plant pathology, entomology, with schedules of control measures, pesticide chemistry, toxicological aspects of crop protection, with epidemiological aspects of diseases and pests, mode of action of pesticides etc. The authors, who are university professors, tried to summarize all of the principles of the above-mentioned requirements in this excellent volume.

First of all they are treating the infecting and damaging agents from a biological point of view (viruses, bacteria fungi, parasitic higher plants, nematodes, arthropodes and vertebrates). The next chapter is on the different symptoms of diseases and damages caused by both microorganisms and pests (mostly insect pests). One chapter is dealing with the infection process and the metabolic aspects of diseases and pests. Mechanism of disease resistance and resistance to the attack of animal parasites are widely discussed, and the paragraph on the genetical aspects of plant-parasite relationships is extremely interesting. The next chapter is on the population dynamic and on epidemiology. The last chapter deals with disease and pest management (chemical as well as biological plant protection, biotechnical management and integrated plant protection).

This book is very well edited and is useful for students primarily interested in plant pathology, entomology, pesticide chemistry and related fields of plant sciences. The progress made in recent decades in the development of theoretical as well as of the practical concepts of plant protection has been so great, that the presentation of plant medicine to the students of present generation in a logical and straightforward way was long overdue. The book on "phytomedicine" fulfilled that requirement.

Z. K.

H. NIRENBERG: *Untersuchungen über die morphologische und biologische Differenzierung in der Fusarium-Sektion Liseola.* (Investigations on the morphological and biological differentiation in the Fusarium section Liseola.) Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft, Berlin—Dahlem, Heft 169, Paul Parey, Berlin—Hamburg 1976. 117 pp. (Price: DM 16.90)

Section Liseola of the genus *Fusarium* was originally erected by Wollenweber for 8 species and varieties with thin-walled macroconidia and oval microconidia, without chlamydospores. Further taxonomic studies of the last thirty years (Snyder and Hansen 1945; Bilay,

1955; Gerlach, 1970; Booth, 1971; Joffe, 1974) led to the conclusion that great species reduction is needed in this section. Hence, the main representative remained *F. moniliforme* and 1 to 3 varieties of this species were generally accepted.

In this study, the taxon *F. moniliforme* is rejected and the section itself is resolved into 7 species and 3 varieties. The results of the taxonomic examinations were based on a worldwide specimen collection including nearly 200 isolates and herbarium specimens from 40 different plant genera. To obtain convincing differentiations among species and varieties, the following criteria were employed: colony and mycelium characters, type of the phialides, form and size of the macro- and microconidia, cardinal temperature, fragrance and colour. If the perfect stage is known, morphological characters of the ascospores were also taken into account. Each taxon is described extensively and supported by numerous photographs and drawings.

Using a representative lot of the isolates seedlings, stalks and cobs of maize, rice seedlings, onions, pine seedlings as well as apple and pear fruits were artificially inoculated in different ways. In general, fusaria of the section Liseola proved to be aggressive, but only weakly virulent. *F. verticillioides* and *F. sacchari* var. *subglutinans* may cause the stalk and ear rot of maize, *F. fujikuroi* is pathogenic to rice, *F. proliferatum* is a causal agent of the fruit rot of apple and pear. On the basis of the literature and from observations on the natural occurrence of the "Liseola" species the phytopathological importance of these fungi are discussed.

This work is a useful tool for mycologists interested in taxonomical problems of the genus *Fusarium* and for plant pathologists who are studying the *Fusarium* diseases of different crops.

L. HORNOK

Microbiology of Aerial Plant Surfaces. Edited by C. H. DICKINSON and T. F. PREECE. Academic Press, London, New York, San Francisco, 1976. 669 p. Price: £ 12.80.

The international meeting of which this volume is the record took place at the University of Leeds in September 1975. The contents record progress in work on the aerial surfaces of plants during the years 1970—1975 and they extend the review provided by the record of the proceeding of the earlier meeting at Newcastle University in 1970.

The book comprises 31 papers provided by more than 40 contributors. Most of the papers deal with ecological systems on aerial surface of plants. Four papers contain studies of biological control of plant pathogens. A valuable paper by BILLING describes the updated problems of the taxonomy of bacteria on the aerial parts of plants. Air pollution, the effects of pests and agrochemicals are well stressed by SMITH and HISLOP. The structure of the surfaces and the plant epicuticular waxes are presented by CUTTER and JEFFREE *et al.*

Contributors to the volume, all specialists in their particular fields, have produced here a worthy successor to Ecology of Leaf Surface Micro-organism. After reading the book it is clear that a considerable amount of information has accumulated on the subject since 1970. On the other hand it is evident that much more information is needed on the interactions of different microorganisms. There is a notable lack on the physiological aspects of microorganisms living together on plant surfaces.

Printing, layout and references are satisfactory and few misprint appear. The book could be recommended for plant pathologists, departmental and institutional libraries.

S. SÜLE

R. K. S. WOOD and A. GRANITI (editors): *Specificity in Plant Diseases.* Plenum Press, New York and London, 1976. 1—354 p.

This book is volume 10 in Series A: Life Sciences of the NATO Advanced Study Institute Series, and contains the papers presented on the interactions that determine the specificity characteristic of diseases of higher plants caused by fungi and bacteria. Virus diseases were not treated in this Symposium, which was held in Alghero (Sardinia), Italy, May 4—17, 1975. In addition to the plant pathological papers, one participant (R. C. HUGHES) lectured on animal membranes as recognition sites and H. F. LINSKENS on specific interactions between higher plants. He also treated analogies between incompatibility and immunology and has shown analogies between the infection processes in plant diseases and the incompatibility reaction in the fertilization of higher plants.

This excellent volume contains presentations by world leaders in their fields with critical reviews and provocative speculations. After each presentation a number of contributions are given but only in titles. The summary of points from contributions and discussions are given after each presentation by the chairman and discussion leader of the session.

After the opening address by A. CICCARONE, the phenomenon of specificity in plant disease was treated by P. W. BRIAN (Cambridge). In a contributed paper HEATH investigated the stages during rust infection at which specificity may be determined. OUCHI pointed out that host cells conditioned by pathogenic races or species are subsequently unable to recognize the incompatible or non-pathogenic mildews as foreign entities and permit successful infection. A. GRANITI discussed tissue and organ specificity in plant diseases, emphesizing the lack of knowledge in this field.

JOHNSON lectured on the genetics of host-parasite interaction and the summary on the discussion was given by ELLINGBOE. Peter DAY discussed the gene functions in host-parasite systems from the point of view of the induced susceptibility hypothesis. Points of comments and contributions have been summarized by PERSON. Following the lecture by HUGHES on animal cell surface membranes as recognition sites, four contributed papers discussed the existence of recognition sites for infectious plant pathogens. ALBERSHEIM's report on cell surfaces and the biochemistry of varietal specific pathogenesis was essentially the same that has been published in Ann. Rev. Plant Physiol. 26, 31—52, 1975. His hypothesis on the inducers (elicitors) of phytoalexins stimulated much discussion. SCHEFFER stated in his summary that there were doubts that this hypothesis will lead to an understanding of host specificity in plant pathogens. In two contributed papers CALLOW discussed the possible role of lectins in recognition and defence in plants. Evidently, more data are necessary to evaluate the role of both "elicitors" and lectins in specific host-parasite interactions. RUDOLPH lectured on the interaction between plants and bacteria. He pointed out that it has not been shown with certainty whether a few dead plant cells are the cause of the resistant reaction or a secondary response. The paper of BUSHNELL on reactions of cytoplasm and organelles treated the recent knowledges on papillae formations and on the role of haustoria in diease resistance. DALY was provocative in discussing his induced susceptibility hypothesis as exemplified with rust diseases. He thinks hormonal changes are causative factors in determining host-parasite specificity. He speculated on resistance that develops if a metabolic switch is not turned on, and suggested that hormones may triggering each switch in susceptible plant. Incompatibility may not involve an active, positive process or mechanism controlling the pathogen. There are situations where parasites simply do not have the proper environment for development. He attacked the hypotheses on the role of activation of protein biosnythesis, formation of antifungal compounds (phytoalexins) and hypersensitivity in disease resistance. The contributed paper of TANI was of special interest. He concluded that senescence rather than juvenescence promotes rust uredospore formation in oat leaves. ROHRINGER lectured on the involvement of nucleic acids in host-parasite specificity. The compound that determines the resistant reaction (in fact the hypersensitive reaction with tissue necrosis) in wheat to stem rust is presumably RNA. This hypothesis was seriously discussed by the session. DEVAY summa-

rized the data available on the protein specificity in plant disease development. The possible role of agglutinins (lectins), and common antigens (protein sharing) was emphesized. WHEELER summarized the new advances on phytotoxins and seriously criticized the results from STROBEL's laboratory on the specificity of *H. sacchari* toxin. DALY's comment was also critical but ALBERSHEIM and STAPLES spoke in support of STROBEL's work that the toxin acts binding to the protein in membranes of susceptible host. It was a general agreement that, if STROBEL's evidence is confirmed, it will provide for the first time at the molecular level a specific mechanism for virulence in the pathogen and a specific mechanism for susceptibility in the host.

The preformed resistance factors were treated by SCHÖNBECK and four contributed papers added new knowledges to the role of saponins, sterols and glucosidase enzymes. KUC's report on phytoalexins was amplified by a number of contributed papers. His result on induced protection of cucumber leaves by prior inoculation with *C. lagenarium* attracted much comment. This protection is systemic in nature and does not related to phytoalexin production. A lecture on the induction of host hypersensitive response was given by SIJPESTEIJN. Hypersensitive reaction caused by bacteria was treated by SEQUEIRA. He made emphasis on the protection (suppression) of the hypersensitive reaction. He called the attention to the wound hormone hypothesis of RYAN. Wounding releases a hormone that induces the accumulation of proteinase inhibitors. Proteinases of pathogens would be inhibited which means that an immune response develops in the plant as a consequence of injury.

The volume is ended with a summing-up paper by WOOD on "Specificity, an assessment".

Professors WOOD and GRANITI made an excellent work editing this book in the present form, which gives a balanced mixture of results, ideas, criticisms, speculations, provocative comments and occasionally perhaps even idiosyncrasy. This volume is highly recommended to all sorts of plant scientists in addition to plant pathologists.

Z. KIRÁLY

Contents

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Lehrbuch der Phytomedizin

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Mensch, Tier und Pflanze sind als Lebewesen eigentümlichen Veränderungen und Abweichungen im Ablauf physiologischer Prozesse unterworfen, die als Krankheiten bezeichnet werden. Die Medizin als wissenschaftliche Arbeit an der Krankheit, an ihrer Diagnose und Therapie, kennt daher drei Disziplinen: die Humanmedizin, die Veterinärmedizin und neuerdings auch die Phytomedizin, deren Aufgabenbereich weit über den der traditionellen Pflanzenpathologie hinausgeht.

So hat sich der Begriff »Phytomedizin« im deutschen Sprachraum nunmehr durchgesetzt und wurde daher von den Autoren für den Titel dieses modernen Lehrbuches gewählt, das aus ihrer täglichen Praxis als Hochschullehrer heraus entstanden und in seiner didaktischen Konzeption auf die Ausbildung des akademischen Nachwuchses ausgerichtet ist. Das Werk vermittelt zugleich die Grundlagen für ein eigenes und aktuelles Berufsbild des Phytomediziners, das den heutigen wissenschaftlichen und praktischen Anforderungen beim Schutz der Pflanzenproduktion, beim Gesundheitsschutz für Produzenten und Verbraucher und den vielfältigen Aufgaben beim Umweltschutz voll entspricht.

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