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Petri Dish-ELISA, a Simple and Economic Technique for Detecting Plant Viruses

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A large number of microtiter plates are needed for mass testing of planting material for viruses in seed certification and plant quarantine. In the case of poorly equipped laboratories, problems with availability of microtiter plates have economic implications for the broad acceptance of enzyme-linked immunosorbent assay (ELISA) for e.g. seed health testing in developing countries. In this experiment the potential of an alternative, cheaper technique was investigated. A conventional indirect ELISA procedure was followed for comparison between the polystyrene solid phases of plastic Petri dishes and microtiter plates for detection of three viruses belonging to the *Tobamovirus, Comovirus* and *Potyvirus* genera. A wax pen was used to divide the inner surface of a polystyrene Petri dish into many circles or squares. The hydrophobic boundaries thus provided were effective in separating 50 µl-drops throughout the ELISA procedure. In comparative assays of serial dilutions of the three viruses, the ELISA in Petri dishes resulted in similar or higher A_{405} values than the ELISA carried out in microtiter plates, suggesting a similar or better protein binding capacity of Petri dishes. The perspectives of this alternative method are briefly discussed.

Keywords: ELISA, microtiter plate, Petri dish, polystyrene.

Since its introduction in plant virology in the late 1970 (Voller et al., 1976; Clark and Adams, 1977) enzyme-linked immunosorbent assay (ELISA) has become the most widely used technique for various kinds of virus detection. Although ELISA puts fewer demands on equipment and facilities than the nucleic acid-based detection methods, performing conventional ELISA still meets some constraints in laboratories in the third world. In developing countries in the tropical and subtropical zones, where a great variety of virus-susceptible crop species are grown and the all-year virus vector pressure is high, management of virus diseases is particularly important. An essential part of this management is the availability and use of healthy planting material through seed certification and plant quarantine inspections, which are often not efficient due to a lack of laboratory equipment and materials. There is therefore a marked need for the development of simple, cheap, accurate and rapid testing methods suitable for large-scale routine use (Lange and Heide, 1986). Some simplified variants of enzyme-linked immunoassay (EIA) have been developed such as dot immunobinding assay (DIBA) using plain paper instead of expensive nitrocellulose membranes (Heide and Lange, 1988) and tissue blotting immunoassay (TBIA) (Lin et al., 1990 and others).

Several variants of ELISA have been developed, but they all, to our knowledge, have in common the need for microtiter plates and a spectrophotometric plate reader, often difficult to procure in the third world because of their high prices and the scarcity of foreign exchange for their import. The maintenance/repair of ELISA readers often also poses a difficulty.

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The experiments described here were undertaken to clarify the potential of a Petri dish-ELISA technique, designed by the authors, and to determine to what extent this technique fulfils the demands for sensitive and accurate routine detection of plant viruses.

Materials and Methods

Antigens and antisera

Dry leaf materials infected with tomato mosaic (ToMV, *Tobamovirus*), cowpea mosaic (CPMV, *Comovirus*) and blackeye cowpea mosaic (BlCMV, *Potyvirus*) were obtained from the virus culture collection of the Danish Government Institute of Seed Pathology (DGISP). The infected tissues, which have been stored over desiccant $CaCl_2$ were ground in the presence of phosphate buffered saline, 0.05M, pH7.2 (PBS) using 1 part of tissue + 9 parts of PBS (w/v). The extracts, considered to correspond closely to original extracts of fresh plant material as regards virus concentration, were centrifuged at 10,000 rpm for 10 min in a Heraus Biofuge-15 centrifuge to remove chloroplasts and plant debris. Series of dilutions were made by further diluting the supernatants from X100 to X25600 in PBS, thus with virus concentrations close to those which could be obtained from serial dilutions of fresh plant tissue. Extracts of healthy leaf material from the same species were prepared in the same manner for use as negative controls at corresponding serial dilutions. Polyclonal antisera of ToMV, CPMV and BlCMV were obtained from the antiserum bank of the DGISP. The antisera were diluted 1/1000 in serum buffer (PBS + 2% polyvinylpyrrolidone + 0.2% chicken egg albumin).

ELISA procedures

An indirect-ELISA procedure, similar to that described by Hobbs et al. (1987) for the DAC (direct antigen coating) method, was followed when comparing the polystyrene solid phases of plastic Petri dishes and microtiter plates (Nunc, Maxisorp™ F=96, Denmark). The comparative experiments were performed simultaneously. Standard polystyrene Petri dishes of 9 cm diam. (Kerial Plast, Denmark) were prepared for ELISA by dividing the inner surface into many circles or squares with a hydrophobic substance, either by use of a DAKO™ pen used for immuno histochemical tests or, even more efficiently, by a wax-based crayon (of the kind used for children's colouring books). A paper template of 45 squares (1x1 cm) placed under the Petri dish was used for convenient dividing and for numbering the positions of the circles/squares. Thus in each Petri dish up to 45 samples could be processed. An orientation point was marked both on the plate edge and the template. Any liquid loaded remained drop-shaped inside the area of the square or circle due to the hydrophobic action of the wax, which made a barrier around the drop *(Fig. 1)*.

Throughout the Petri dish procedure, 50 µl of each of the reactants were loaded carefully into the centre of each circle or square using a precision pipette. Eight replicates were made for each dilution. After loading the antigen dilutions, the Petri dish was incubated under a lid for 1 h at 37 °C. Then the Petri dish was unloaded in one swift movement

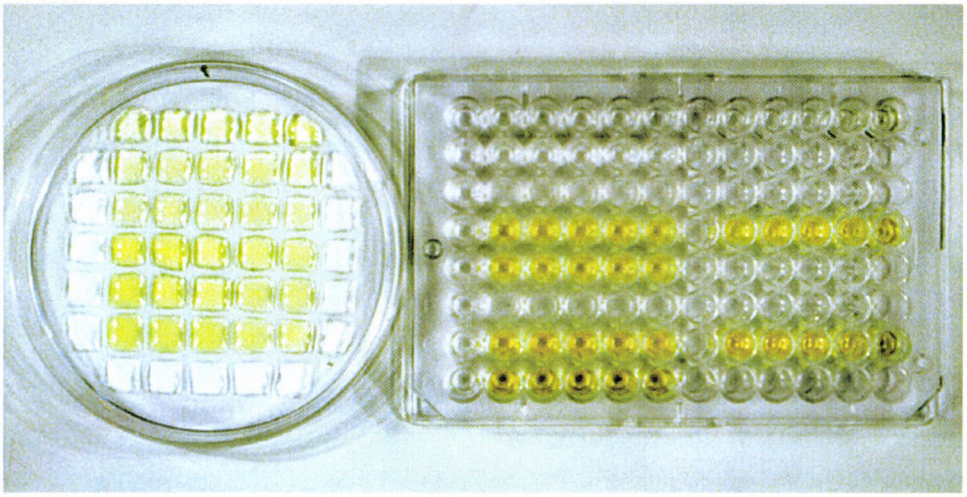


Fig. 1. ELISA carried out in polystyrene Petri dish and microtiter plate. As shown, reagents remain drop-shaped inside the squares drawn by a hydrophobic wax pen

and rinsed with PBST (PBS + 0.02% KCl + 0.05% tween 20) followed by covering the inner surface with three changes of PBST. Washing was done by slowly rotating the buffer inside the plate for 3 min between each change. After the wash the Petri dish was gently blotted by touching the inner surface of the plate with a piece of filter paper without causing any damage to the hydrophobic wax lines. The two next reactants, i.e. dilutions of crude specific antisera and swine anti-rabbit serum enzyme conjugate, were loaded into the circles/squares and allowed to incubate under a lid, each for 1 h at 37 °C. Washing as described above followed both incubations. Finally, each square or circle was loaded with substrate solution (approx. 0.4 mg/ml of p-nitro phenyl phosphate in substrate buffer). In the experiments comparing Petri dish-ELISA with microtiter plate-ELISA, each sap dilution was loaded and processed in four replicates.

A parallel procedure in microtiter plates was carried out, using 100 µl of each reactant and three replicates per dilution, but otherwise according to Hobbs et al. (1987) and with the same concentrations, incubation times and temperatures as used for Petri dishes.

After 30 min incubation at room temperature the substrate was examined visually for a yellow colour development indicating positive reactions, and after a further 30 min the substrate reaction was stopped both in Petri dishes and microtiter plates by adding 5 µl of a 3 M NaCl solution to each square/circle and each well. Then the reactions in microtiter plate wells were read spectrophotometrically by an ELISA reader (Titertek Multiscan™) at 405 nm wavelength. To read the absorbance values of Petri dish reactions by ELISA reader, samples from all the 8 replicates of each dilution were transferred into wells of a clean microtiter plate by a precision pipette. Three sets of Petri dish replicates per dilution were combined in this way making three wells of 100 µl. All sets of Petri dish replicates were combined in this way, and finally a comparison between the absorbance values from the microtiter plate ELISA and the Petri dish-ELISA for detecting the three viruses was made.

Results

The data presented are reading averages of replicates from two experiments, performed in both Petri dish and microtiter plate. Absorbance values representing ELISA reactions of serially diluted ToMV, CPMV, and BlCMV-infected samples and healthy controls are presented in *Table 1*, and for convenient comparison also graphically in *Fig. 2.* Antigen-antibody reactions for all dilutions of infected samples were positive in the polystyrene Petri dishes as well as in the microtiter plates processed in parallel. Even at the highest dilution (1:25600) of infected tissue in buffer the viruses could be detected in both systems, still with absorbance values 7–16 times higher than in the corresponding dilution of healthy material. Dilutions ranging to the limit of detection and beyond were not included in these experiments, the purpose of which was to compare the sensitivity of two detection systems.

Table 1 (A)

Comparison between ELISA in polystyrene Petri dish and microtiter plate for detection of tomato mosaic virus (average absorbance values)

Antigen Dilution	ToMV *Tobamovirus*			
	Petri dish		Microtiter plate	
	H*	I	H	I
	**			
1/100	0.086 *0.002*	2.010 *0.004*	0.061 *0.003*	1.134 *0.005*
1/200	0.075 *0.002*	1.965 *0.004*	0.051 *0.001*	0.795 *0.006*
1/400	0.074 *0.003*	1.838 *0.005*	0.049 *0.001*	0.777 *0.003*
1/800	0.071 *0.001*	1.767 *0.004*	0.047 *0.001*	0.722 *0.001*
1/1600	0.071 *0.001*	1.662 *0.003*	0.046 *0.002*	0.618 *0.006*
1/3200	0.068 *0.002*	1.311 *0.004*	0.045 *0.001*	0.599 *0.008*
1/6400	0.065 *0.003*	1.113 *0.005*	0.045 *0.001*	0.542 *0.003*
1/12800	0.051 *0.001*	0.829 *0.006*	0.039 *0.001*	0.530 *0.003*
1/25600	0.040 *0.001*	0.665 *0.002*	0.011 *0.001*	0.425 *0.004*

* H = healthy, I = infected,

** values are the standard deviation within each mean of absorbance readings.

The data presented show that the A_{405} values of infected samples for the 3 viruses at the dilutions 1:200–1:6400 were often one and a half and sometimes two times higher for ELISA performed on plastic Petri dishes compared with those of microtiter plates. Readings tended to be more similar at higher dilutions (1:12800 and 1:25600) for infected samples with ToMV and CPMV *(Table 1* and *Fig. 2).*

The A_{405} values for healthy samples were proportionally low in both systems. However, the ratios of corresponding A_{405} values for infected samples/A_{405} values for healthy samples, indicating the specificity and sensitivity of the assays, were higher for the Petri dish (PD) system than for the microtiter plate (MP) system. E.g. for BlCMV the

Table 1 (B)

Comparison between ELISA in polystyrene Petri dish and microtiter plate for detection of cowpea mosaic virus (average absorbance values)

Antigen Dilution	CPMV *Comovirus*			
	Petri dish		Microtiter plate	
	H*	I	H	I
1/100	0.064 *0.001***	2.181 *0.005*	0.082 *0.001*	1.638 *0.005*
1/200	0.058 *0.002*	1.846 *0.007*	0.072 *0.003*	1.452 *0.005*
1/400	0.057 *0.001*	1.604 *0.005*	0.054 *0.003*	1.423 *0.004*
1/800	0.052 *0.001*	1.446 *0.007*	0.053 *0.003*	0.963 *0.006*
1/1600	0.051 *0.001*	1.323 *0.004*	0.050 *0.002*	0.884 *0.005*
1/3200	0.050 *0.001*	1.121 *0.002*	0.049 *0.002*	0.660 *0.004*
1/6400	0.049 *0.001*	0.880 *0.003*	0.044 *0.003*	0.618 *0.006*
1/12800	0.049 *0.001*	0.619 *0.008*	0.043 *0.001*	0.529 *0.001*
1/25600	0.049 *0.001*	0.567 *0.008*	0.043 *0.001*	0.417 *0.001*

* H = healthy, I = infected,

** values are the standard deviation within each mean of absorbance readings.

Table 1 (C)

Comparison between ELISA in polystyrene Petri dish and microtiter plate for detection of blackeye cowpea mosaic virus (average absorbance values)

Antigen Dilution	BICMV *Potyvirus*			
	Petri dish		Microtiter plate	
	H*	I	H	I
1/100	0.064 *0.005***	2.327 *0.008*	0.082 *0.001*	1.684 *0.005*
1/200	0.058 *0.002*	2.179 *0.006*	0.072 *0.001*	1.272 *0.003*
1/400	0.057 *0.002*	2.107 *0.004*	0.054 *0.001*	1.159 *0.002*
1/800	0.052 *0.001*	1.943 *0.004*	0.053 *0.001*	1.009 *0.002*
1/1600	0.051 *0.001*	1.759 *0.006*	0.050 *0.002*	0.993 *0.004*
1/3200	0.050 *0.001*	1.552 *0.009*	0.049 *0.002*	0.725 *0.001*
1/6400	0.049 *0.001*	1.149 *0.003*	0.044 *0.001*	0.560 *0.002*
1/12800	0.049 *0.001*	1.086 *0.007*	0.043 *0.001*	0.509 *0.002*
1/25600	0.049 *0.001*	0.599 *0.005*	0.043 *0.001*	0.293 *0.001*

* H = healthy, I = infected,

** values are the standard deviation within each mean of absorbance readings.

average ratio was 15 in the MP, but 30 in the PD, and for CPMV 17 and 24, respectively. The ratios of the corresponding infected/healthy values in *Table 1* varied from 38 (2.179/0.058 for BICMV in PD at dil. 200) to 7 (0.293/0.043 for BICMV in MP at dil. 25600). The results demonstrated improved detectability in the PD system regardless of virion types that in this case represented three virus genera. The visual evaluation of the reactions in the PD system was at least as easy as of those in the MP system *(Fig. 1)*.

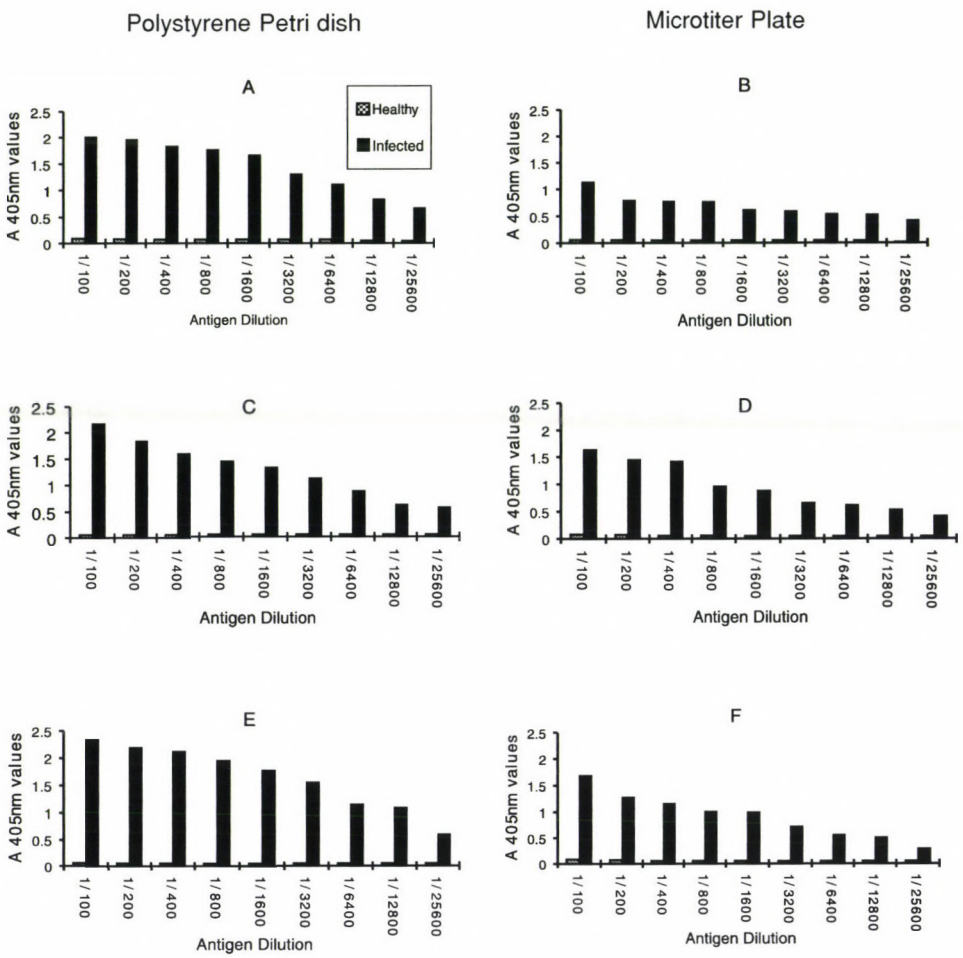


Fig. 2. Comparison between ELISA carried out in polystyrene Petri dish and microtiter plate for detection of 3 viruses belonging to different genera. A–B for ToMV (tobamovirus), C–D for CPMV (comovirus) and E–F for BICMV (potyvirus)

Discussion

A comparison of the absorption values of the two methods suggests a similar or even better protein-binding ability of the polystyrene of the Petri dishes used here than that of the microtiter plates. This was reproduced in a number of comparative tests, made before and after those reported here, and which included also viruses like cucumber mosaic cucumovirus and tomato ringspot nepovirus (data not shown). The performance of different batches or other brands of Petri dishes was not investigated.

An obvious risk one could expect from the Petri dish method is an unintended mixing of reactants between the drops. However, this occurred surprisingly rarely during the several tests made prior to, as well as in, the actual experiments. Provided that the wax boundaries were made accurately and any drops of remaining buffer after wash were removed before adding the subsequent reactant, none of the reactive drops merged. Also, no mixing took place during normal handling, observing that heavy shaking or shocks were avoided when moving the Petri dishes e.g. to and from the incubator.

The colour intensity was easier to rate visually in the drops on the Petri dish bottom than in the wells of the microtiter plate, where disturbing effects of reflection in the walls of the wells occurred. For both systems a light box or a white paper was used as background. To ensure correct conclusions without a spectrophotometric reader it is, however, particularly for the Petri dish method recommendable, prior to each virus/host/test reagent combination in the routine mass-testing to be undertaken, to adjust the test set-up in such a way that clear-cut positive and negative reactions will be obtained. This means choosing the optimum dilutions of extract, antiserum and conjugate, and, if composite samples are tested, not least adjusting the maximum number of plants or seeds to be represented in each sample, allowing the clear-cut detection of one infected plant or seed, even if it has a low virus content, among X healthy ones.

The use of other enzyme/substrate systems such as penicillinase/penicillin using bromothymol blue as the colour indicator (Sudarshana and Reddy, 1989; Abraham and Albrechtsen, 2001, in press) which could improve the visual rating in Petri dish-ELISA because of the clear change from blue to yellow in the presence of virus, should be investigated. Also the performance of other ELISA procedures in the Petri dish assay, such as double-antibody-sandwich (DAS) or triple-antibody-sandwich (TAS) ELISAs should be evaluated.

The main advantage of the Petri dish-ELISA method is the reduced costs. An ELISA reader is not needed, only 50 µl or less is required of each reactant compared to 100 or 200 µl normally used in microtiter plates, and if three Petri dishes replace one microtiter plate, the cost of solid support will be reduced 5- to 8-fold. Compared with the use of microtiter plates Petri dish-ELISA requires, however, some preparative work and more accuracy in the application of the reactants and thereby a slight increase in time/labour, which should be included when calculating the over-all cost.

An alternative way to reduce costs could be the re-use of microtiter plates as suggested by e.g. Manandhar et al. (1996). In this work cleaning microtiter plates by a detergent following a prescribed procedure allowed the safe use of each plate up to four times. It will, however, require the procurement of microtiter plates.

The results indicate that ELISA can be performed as sensitively and specifically in a polystyrene Petri dish as in a microtiter plate. Virions belonging to three virus genera were equally well detected, suggesting that the detectability of different virion types in polystyrene Petri dishes is comparable to that of microtiter plates. The simplicity and the reduced costs may make the method applicable for large-scale testing in poorly equipped laboratories.

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Biological Decline of *Solanum nigrum* L. Due to Tobacco Mosaic *Tobamovirus* (TMV) Infection I. Growth and Nutrient Uptake*

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The aim of our study was to examine the effect of tobacco mosaic *tobamovirus* (TMV) infection on the growth and nutrient content of *S. nigrum* leaves. TMV infection significantly reduced the height, the fresh and dry weight of both the shoots and roots. The height of the shoots was reduced by 53% as compared to control. Reduction in fresh and dry weight of shoots and roots due to virus infection varied between 78 and 82%. There was no significant difference in the nitrogen (N), phosphorus (P) and calcium (Ca) content of the healthy and virus infected leaves of *S. nigrum*. The sodium (Na), magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn) and copper (Cu) content of the leaves significantly reduced due to virus infection. Opposite effect was observed in case of potassium (K) content, which were considerably enhanced in the TMV infected leaves.

Keywords: *Solanum nigrum*, TMV, tobacco mosaic *tobamovirus*.

Weeds can influence the quality and quantity of crops not only in a direct way (e.g. by competing for nutrient and water uptake), but also as alternative hosts of various pathogens. Alternative hosts serve as food for the vectors of viruses, while the seeds and vegetative reproductive organs of certain weed species play an important role in the epidemiology and overwintering of viruses. The positive side of the indirect effect is the so-called "bioherbicide strategy", when natural enemies of weeds (pests, pathogens) are used for suppression of a weed population. Biological decline of weeds due to virus infection have been recently studied. It was stated that germination and seed viability of *Chenopodium* seeds from the sowbane mosaic *sobemovirus* (SoMV) infected plants was strongly reduced, therefore virus infection may contribute indirectly to the reduction of weed seed bank of the soil (Kazinczi et al., 1997, 2000; Kazinczi and Horváth, 1998a). Both cucumber mosaic *cucumovirus* (CMV) and henbane mosaic *potyvirus* (HeMV) retarded the growth of *Datura stramonium*. Besides this, the disturbance of water relation of *D. stramonium* and *S. nigrum* was observed due to HeMV and CMV infection, respectively. Additionally, CMV infection significantly reduced the photosynthetic pigment content of *Datura stramonium* leaves (Kazinczi et al., 1996, 1998c).

A lot of study have dealt with the effect of different nutrients on the development of pathogens and disease symptoms on crops. The effect of different nutrients on the occurrence of disease symptoms and development of the pathogens may vary greatly, depending on the type and dosage of fertilizers, the ratio of the different elements, host plant and pathogen species and the phase of the development of the pathogens (Standaert et al.,

*Dedicated to Prof. Dr. J. Horváth, on the occasion of his 65th birthday

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1978; Shaikh and Ghaffar, 1986; Cloud and Rupe, 1994; Reuveni et al., 1995). Viral infection may result in an increase of the exudation of nutrients from roots of infected hosts, which may enhance the potentiality of root fungal pathogens of those hosts (Beute and Lockwood, 1968). In spite the fact that a lot of study have dealt with the effect of nutrients on the development of the pathogens and plant disease, little data is available about the changing of the nutrient content of the host plants due to the different pathogens. Kazinczi et al. (1998a, b) studied the effect of *Macrophomina phaseolina* and viruses on the nutrient content of pepper leaves. It was concluded that the infections have played an important role in the early nutrient uptake of pepper and after flowering no significant reduction was observed. In case of several mixed infection with viruses and fungus the reduction in the nutrient content of the pepper leaves was greater. Although the nutrient content of the weeds was intensively studied by different authors (Tölgyesi, 1969; Debreczeni, 1988; Lehoczky, 1988; Kazinczi, 1993; Lukács et al., 1998) comparative investigations have not yet been available, regarding the healthy and virus infected plants.

Members of the family Solanaceae have economical and also virological importance. *S. nigrum* is reported as a weed in 37 crops and in 73 countries (Holm et al., 1977). In Hungary, under moist environments and high fertility of soils in late-sown cultures – especially in vegetables – it is considered to be a noxious weed (Hunyadi et al., 1998). It is tolerant to the dinitroaniline type herbicides (Kádár, 1983), which are frequently used in vegetable and triazine resistant biotype of *S. nigrum* is also known (Garcia-Baudin and Aguirre, 1983). *S. nigrum* is known as a virophilous species and host of 43 viruses (Mariappan et al., 1973; Davis and Allen, 1975; Schmelzer and Wolf, 1977; Wilson et al., 1981; Dimitrievič, 1985; Alegbejo, 1987; Farrel and Stufkens, 1988; Daniel and Tsai, 1990; Kazinczi and Horváth, 1998b). It is natural hosts of *Polymyxa betae* fungi, which is the main vector of a serious sugarbeet virus, beet necrotic yellow vein *benyvirus* (Vurbanov and Kroumov, 1989). Kistah et al. (1986) studied physiological and ultrastructural changes of *S. nigrum* due to potato Y *potyvirus* (PVY) infection. On the basis of their observation the chlorophyll content of the leaves decreased, respiration rate increased and ultrastructural changes of mitochondrium, chloroplast and cell wall were also observed.

The aim of our study was to examine the effect of TMV infection on the growth and nutrient content of *S. nigrum* leaves.

Materials and Methods

Seeds of *S. nigrum* were sown in sterilized boxes in the virological glasshouse free of vectors. The seedlings were planted in plastic pots (28 cm in diameter), containing a soil mixture of sand (pH: 6.96, humus%: 0.27): peat (pH: 6.78, humus %: 9.98) 1:3. *S. nigrum* plants were mechanically inoculated at 6–8 leaf stage with U1 strain of tobacco mosaic *tobamovirus* (TMV/U1). Previously the virus was propagated on *Nicotiana tabacum* L. cv. Samsun. There were four plants in a pot and eight replicates of control and TMV infected plants. Tests continued for 90 days, when the fresh and dry weight both of the shoots and roots of plants were measured. To determine the nutrient content, leaf samples were collected at flowering (at the end of July). The nitrogen (N), phosphorus

(P), potassium (K) and sodium (Na) content was determined after destruction with sulphuric acid by a photometer. K and Na content was determined by flame photometer. After hydrochloric hydrolysis, atomadsorption spectrophotometer was used to determine the calcium (Ca), magnesium (Mg), zinc (Zn), copper (Cu), manganese (Mn) and iron (Fe) content of the leaves.

Results and Discussion

TMV infection significantly reduced the height, the fresh and dry weight of both the shoots and roots. The height of the shoots was reduced by 53% as compared to control. Reduction in fresh and dry weight of shoots and roots due to virus infection varied between 78 and 82% *(Table 1).*

Table 1

The effect of tobacco mosaic *tobamovirus* (TMV) infection on the growth of *Solanum nigrum* L.

	Height (cm)	Fresh weight (g)		Dry weight (g)	
		Shoot	Root	Shoot	Root
Control	88(4)*	43.2(1.7)	5.5(1)	4.2(0.9)	0.58(0.19)
TMV	41(7)	9.5(4.6)	0.98(0.3)	0.88(0.60)	0.13(0.10)
$LSD_{5\%}$	3.32	3.66	0.78	1.44	0.25

*numbers in brackets indicate the standard error

There was no significant difference in the nitrogen (N), phosphorus (P) and calcium (Ca) content of the healthy and virus infected leaves of *S. nigrum.* The sodium (Na), magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn) and copper (Cu) content of the leaves significantly reduced due to virus infection. Opposite effect was observed in case of potassium (K) content, which were considerably enhanced in the TMV infected leaves *(Figs 1, 2).* The physiological and biochemical background of this unexpected observation is not yet clear. In a previous experiment viruses alone did not decrease considerably the K content of pepper leaves, while significant reduction of K content was observed due to complex infection of *M. phaseolina* and viruses (Kazinczi et al., 1998b).

Our results underpin the previous statement that viruses unfavourably influence physiological processes of the weeds therefore – in indirect way – viruses may contribute to the reduction of their competitive ability. Previously we have proved that CMV infection reduced the drought resistance of *S. nigrum* as compared to the healthy ones (Kazinczi et al., 1998c). Considering that formerly the physiological examinations of the virus diseased plants were connected mainly with the cultivated plants, therefore our present work supplies new data to the biological decline of the virus diseased weed plants.

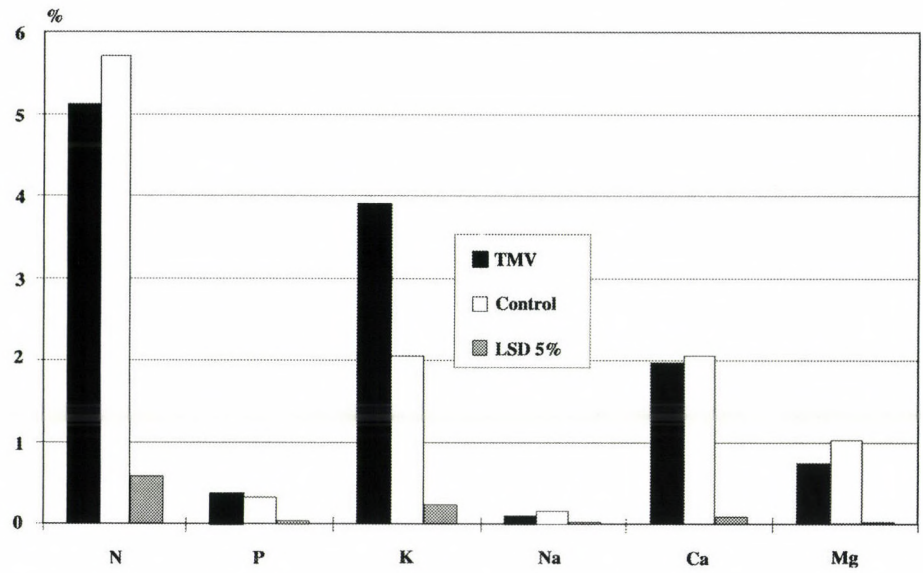


Fig. 1. The effect TMV infection on the nitrogen (N), phosphorus (P), potassium (K), sodium (Na), calcium (Ca) and magnesium (Mg) content of *Solanum nigrum* leaves

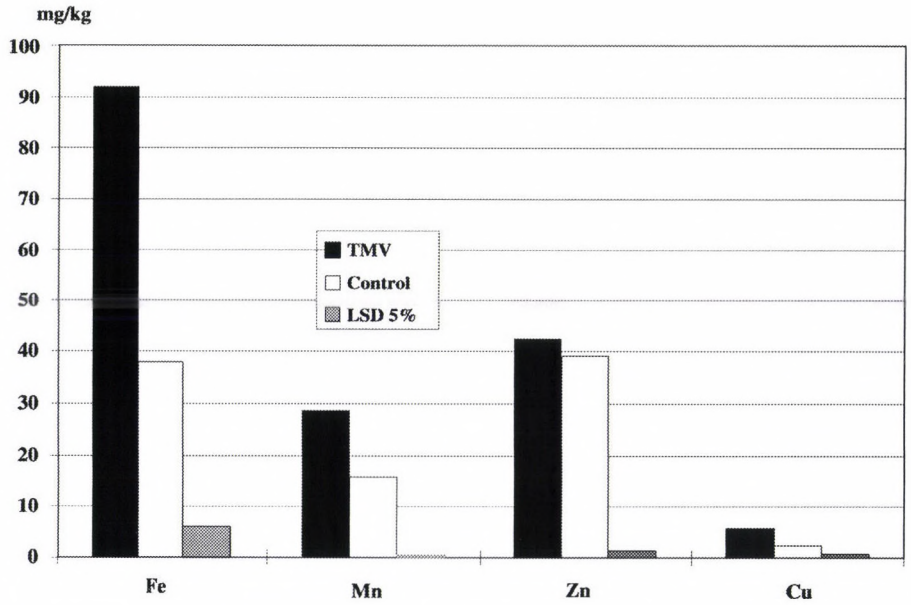


Fig. 2. The effect of TMV infection on the iron (Fe), manganese (Mn), zinc (Zn) and copper (Cu) content of *Solanum Nigrum* leaves

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Photosynthetic Alterations of Virus Infected Plants*

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The most obvious symptom of systemic virus infection is the mosaic pattern of the leaves. Yellowing, chlorosis is also frequent and characteristic sign of the altered photosynthetic activity. Virus infection effects photosynthesis in a complex manner, depending on the particular host-virus combination. The symptoms are basically different in the incompatible or the compatible host-virus interaction.

Keywords: Photosynthesis, virus infection, biotic stress.

Photosynthetic changes in incompatible host-virus interaction

Virus infection often causes local lesions in resistant plants. The development of local lesions in hypersensitive reaction (HR) was described in details by electron microscopy, based on the time course of the appearance of symptoms (Weintraub and Ragetli, 1964). The ultrastructure of the lesion and the surrounding cell layers and the role of the active zone in the localisation of the infection was highlighted (Israel and Ross, 1967).

In tobacco mosaic *tobamovirus* (TMV) infected *N. glutinosa* leaves the size and number of the starch grains increased in the chloroplasts following the infection (Weintraub and Ragetli, 1964). One day after the infection plasmolysis occurred, and first the cytoplasm and later the chloroplast membranes completely disappeared. In the remains of the chloroplasts large vesicles were formed, the number of mitochondria increased and their structure changed. Finally the whole cell structure collapsed and its content disintegrated. All these alterations were consequences of the loss of osmotic regulation, and have been related to the so-called 'starch-lesions' described by Holmes (1931).

Israel and Ross (1967) have studied the cells of necrotic lesions, the tissues in the direct vicinity (active zone), and the outer visually unaffected cells. The cells in the lesions contained very few cell organelles; starch grains, disrupted thylakoid membranes, displaced ribosomes. The cells of the active zone showed increased metabolic activity. The number of chloroplasts increased due to their frequent division. Similar alterations were described in the case of another host plant (*Phaseolus vulgaris* var. Pinto) following TMV infection (Spencer and Kimmins, 1971).

The phenomena of chloroplast swelling and abruption in HR were examined by da Graça and Martin (1975) in TMV infected *N. tabacum* cv. "Samsun NN" plants. The primary symptom of HR was the change in the permeability of membranes. The oxidative burst, the increase in peroxidase and lipoxigenase activity lead to the brake up of membranes (Weststeijn, 1978; Dhindsa et al., 1981; Sutherland, 1991).

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In the lesions of potato virus *S carlavirus* (PVS) infected *Chenopodium quinoa* Shukla and Hiruki (1975) often found cytoplasmic invaginations containing ribosomes in the chloroplasts and in the stroma, the accumulation of fitoferritin, which was not specific to the PVS infection, because other viruses or certain stress factors cause the same symptoms (Rana et al., 1989).

Photosynthetic changes in compatible host-virus interaction

For the synthesis of virions the parasite uses the metabolites and energy of the host plant, which are produced in the course of photosynthesis. It is evident that the physiological processes of the virus and the plant are linked in complex ways, the structure and the function of the chloroplasts are disturbed (Zaitlin and Hull, 1987).

Changes in the ultrastructure of chloroplasts

The chlorosis of leaf tissues in the compatible host parasite interactions can be traced back to different causes. The number, size or chlorophyll content of the chloroplasts may decrease (Tu et al., 1967). Their shape, inner membrane structure may alter to some degrees, some of their metabolites may accumulate, etc. (Goodman et al., 1986). Often the starch grains enlarge, or accumulate for example in the case of the cauliflower mosaic *caulimovirus* (CaMV) infected cabbage plants (Conti et al., 1972). In several other combinations the appearance of large starch grains, the disappearance of thylakoid, stroma and grana membranes and the increase of plastoglobuli (Koiwa et al., 1989; Russo and Martelli, 1981) is similar to the chloroplast to chromoplast transformation (Tomlinson and Webb, 1977; Schuchalter-Eicke and Jeske, 1983), or to the phenomena of senescence. This idea is underlined by the fact that the application of a citokinin type substance (karbendazim) in the leaves of lettuce infected with beet western yellows *betaluteovirus,* (BWYV) caused the suppression of symptoms (Tomlinson and Webb, 1977). Schuchalter-Eicke and Jeske (1983) studied the seasonal rhythm of chloroplast symptoms in abutilon mosaic *bigeminivirus* (AbMV) infected *Abutilon* sp. plants, which can also be correlated with senescence. The microscopic changes reflected the influence of light intensity and season on the lighter/darker mosaic pattern: in summer the inner membrane structure almost completely disintegrated, showed vesicular, tubular or myelin-like structure, while in winter prolamellar body-like structures developed in the plastids, which resulted in the reorganisation of grana. According to the authors the virus regulates the chloroplast/chromoplast transition by switching the organelle's coded 'program' on and off.

The effect of virus infection on the development and differentiation of plastids was summarised in the study of cucumber mosaic *cucumovirus* (CMV) infected tobacco leaves (Ehara and Misawa, 1975). Depending on the time of inoculation, the plastids differentiated irregularly. The ratio of degenerated plastids changed with the time course of symptom development. The myelin-like or tubular structure was not formed by the disruption of the existing lamellae, but by their irregular development. In the case of BSMV infected etiolated barley seedlings we reported not only the accelerated senescence but the inhibition of chlorophyll biosynthesis (Almási et al., 2000).

The presence of vesicles within or attached to plastids was reported by Hibino et al. (1974a,b). In tobacco leaves infected with TMV U5 strain it was proved by autoradiography that the chloroplasts do not take part in the biosynthesis of virions, which means that the vesicles do not play any role in virus replication (Betto et al., 1972). This was also regarded as a secondary effect in tomato spotted wilt *tospovirus* (TSWV) infected tobacco leaves (Mohamed, 1973).

In contrast, the replication of *tymovirus* genus is related to the chloroplasts. The swelling of the chloroplasts and the formation of small vesicles were reported in chinese cabbage infected with turnip yellow mosaic *tymovirus* (TYMV) (Ushiyama and Matthews, 1970). The plastids became globular, the number of vesicles in the plastids increased and they formed clusters covered by endoplasmic reticulum (ER) from the cytoplasmic side (Hatta and Matthews, 1974). The region between the ER and the plastid envelope became electronlucent, and the ER disappeared. Then the plastids clustered and virions were observable in the zone between the plastids. In the vesicles of the inner surface of the envelope, double stranded nucleic acids were visible, which proved to be the replicative form of viral RNA. Based on these results it was supposed that these vesicles serve as sites of viral replication, then the viral RNA translocates into the cytoplasm where the viral proteins are synthesised, and finally the assembly of the virions is completed. These peripheral vesicles open into the cytoplasm with short necks (channels) (Matthews, 1977). Similar changes were noted for leaves of wild cucumber (Marah oreganus) infected with wild cucumber mosaic *tymovirus* (WCMV) (Allen, 1971), where virions were also present in the stroma as well as in the cytoplasmic invaginations.

Abnormal vesiculated plastids were observed in susceptible host plants (barley, wheat) infected with barley stripe mosaic *hordeivirus* (BSMV). The grana of the deformed, swollen plastids had an unusual structure. Between the inner and outer membranes of the envelope single membrane bound vesicles were apparent (Carroll, 1970; Jackson et al., 1989). In the peripheral vesicles of the chloroplasts Lin and Langenberg (1985) had observed the replicative form of viral dsRNA, so the plastids could be related to virus synthesis. Symptom development was categorised into timecourse stages, such as primary, secondary acute (inoculated leaves) and chronic phase (systemic leaves) (Mc Kinney and Greeley, 1965). In yellow tissues of the primary acute phase plastids were often found with translucent stroma, which lacked ribosomes. Their thylakoids were irregularly twisted, forming a tubular reticulum with enlarged grana. In the secondary acute phase the degradation of plastids continued until the death of the cells. The young plastids of the chronic phase contained irregular thylakoids, small, few lamellar grana, which sometimes vesiculated (Mc Mullen et al., 1978). The reason for the development of a mosaic pattern is the clustered layout of the chloroplasts that have been damaged to various degrees. Chalcroft and Matthews (1967) had thought that in the dividing cells of chinese cabbage infected with TYMV the spontaneously mutating virus strains of different pathogenicity end up in different daughter cells. The symptom development was regarded by Gardner (1967) as the result of the different tolerance level of the host cells. Mc Mullen et al. (1978) had proposed that the virus regulates the phenotype of the plastids through the nuclear or plastidial genom. In contrast, in the case of systemic infection of barley yellow mosaic *bymovirus* (BYMV) there was a linear correlation between the virus concentration and symptom severity (Huth et al., 1984).

In certain host-parasite interactions the presence of unusually large grana ("supergrana") had been observed (Šarič and Wrischer, 1975; Sjolund and Smith, 1974). By freeze fracture method replica were made from the granal surface, and based on the number and size of the contained particles the damage of photosystem II (PSII) was determined.

Changes in the chlorophyll protein complexes and chloroplast proteins

Virus infected monocotyledons (e.g. barley infected with BSMV or WSMV) contained reduced amount of chlorophyll protein complexes compared to the healthy controls (White and Brakke, 1983). Koiwa et al. (1992) supposed that virus infection inhibits PSII activity selectively by the decomposition of light harvesting antenna complex of PSII (LHCII), based on their findings on the ratio of particles in the surface of the thylakoid membranes in tomato plants infected with TMV. Similarly, *Eupatorium makinoi* leaves infected with tobacco leaf curl *geminivirus* (TLCV) the amount of LHCII decreased in the chloroplasts (Funayama et al., 1997a,b). The chlorophyll protein complexes and the protein content of the thylakoids in peanut infected with peanut green mosaic *potyvirus* (PGMV) had been altered in parallel to the symptom development (Naidu et al., 1984a). The complexes reduced to the greatest extent were identified by SDS polyacrylamid gel electrophoresis (PAGE). All these components belonged to PSII and LHCII: namely the 47 kD chlorophyll protein, the 23 kD LHCII chlorophyll protein and the α-, β-subunits of the ATP synthase). There were no significant differences in PSI reaction centre, while in LHCI the amount of the chlorophyll protein complexes slightly increased. In our experiments we detected the loss of chlorophyll protein complexes of both photosystems (i.e. PSII and PSI) (unpublished data). In TYMV infected chinese cabbage chloroplasts the antenna complexes (LHCII and LHCI) decreased to the greatest extent, while in TSWV infected *Nicotiana benthamiana* chloroplasts the complexes belonging to the reaction centers (PSII CC and PSI RC) were mostly affected. Later in a more sophisticated system (by improved PAGE techniques) Naidu et al. (1986) detected the loss of 14, 18, 19, 23 and 33 kD proteins. The 23 and 33 kD proteins belong to the oxygen evolving complex (OEC) of PSII, and their changes showed a good correlation with the decreased efficiency of O_2 evolution. Takahashi et al. (1991) determined the 22, 23 and 24 kD proteins of OEC from tobacco leaves infected with CMV separately by using two-dimensional gels. They supposed these three proteins to be the products of the same small multigen family. The amount of the 22 and 23 kD proteins had started to decrease at the time of the appearance of chlorotic spots, while the changes in the amount of the 24 kD and 33 kD proteins which induce more severe symptoms started only after that (Takahashi and Ehara, 1992). To sum up all these results the authors proposed, that the environmental factors do not induce or suppress the distinct elements of certain gene families in the same manner or at the same time. The inhibition of OEC is not a secondary consequence of the symptom development, but it is rather connected with the primary molecular processes of the infection.

Among the stroma proteins the loss of the small subunit of ribulose-1,5-bisphosphate-carboxylase-oxygenase (Rubisco) enzyme was reported in the chlorotic tissues of cucumber plants infected with CMV (Ziemieczki and Wood, 1975), and in the chloroplasts of tobacco leaves infected with TSWV together with the reduced level of 70 S ribosomes (Mohamed and Randles, 1972).

In parallel to the decrease in chlorophyll content a significant increase was measured in the enzyme activity of the chlorophyllase which catalyses the dephytilisation of the chlorophyll molecules (Bailiss, 1970). It was interpreted as the consequence of the release of chlorophyllase enzyme bound to the chloroplast inner membrane following the disrupture of the chloroplasts.

Effect of virus infection on the photosynthetic electron transport

Chlorotic leaves of tobacco infected with tobacco etch *potyvirus* (TEV) showed a decreased level of photosynthetic activity compared to control plants. The light reaction (Hill reaction and photophosphorylation) of isolated chloroplasts showed similar efficiency (Hopkins and Hampton, 1969a). Hodgson et al. (1989) described the selective inhibition of PSII in spinach infected with TMV. The fluorescence kinetic parameters measured in the thylakoids of mosaic pattern leaves showed considerable deviance. The Fv/Fm ratio (the potential photosynthetic efficiency parameter of PSII) significantly decreased to the values of the uninfected DCMU treated leaves. The O_2-evolution of thylakoids was lower than in the healthy control, and this was the primary result of PSII inhibition. The PSI electrontransport was unaffected. The chlorophyll a/b ratio slightly decreased. From the above results the authors concluded that in the symptomatic leaves of virus-infected plants the cyclic phosphorylation and ATP synthesis dominates over the non-cyclic electrontransport that results in $NADP^+$ reduction. In TYMV inoculated leaves of chinese cabbage both the cyclic and the non-cyclic phosphorylation is more intensive during the period of active virus synthesis (Goffeau and Bové, 1965). When the virus concentration reaches its maximum in the plant, in the chloroplasts of systemically infected leaves the previous activity is depressed, the amount of ATP is significantly lower than in the control. Therefore Goffeau and Bové (1965) considered that the ATP formed by photosynthesis as energy source is directly used for the virus protein and nucleic acid synthesis. Kano (1985) drew the same conclusion. Net photosynthesis, chlorophyll content and the ratio of chlorophyll a/b decreased in peanut leaves infected with PGMV (Naidu et al., 1984b). The loss of PSII activity was more pronounced than that of PSI. Both cyclic and non-cyclic photophosphorylation was inhibited, although still functioning.

Funayama et al. (1997b) found higher chlorophyll a/b ratio in the leaves of a *geminivirus* infected *Eupatorium makinoi.* The net photosynthesis measured at saturating light intensity (Pm) and Fv/Fm showed lower values with the increasing Fm. In virus infected plants the proportion of the β reaction centers that have smaller antennae related to the all PSII centers proved to be much higher than in the healthy plants. Energy distribution balance between PSII and PSI broke down (Funayama et al., 1998).

In dark-adapted leaves of *N. benthamiana* plants infected with PMMV-S, PMMV-I or with the hybrid viruses constructed of them. Rahoutei et al. (1998) detected a considerable increase of the non-photochemical quenching (NPQ) while photochemical quenching (qP) decreased. These changes show the diminished ability for energy capture of PSII reaction centers in virus infected plants, and they function as photoprotective mechanisms to irradiate excess energy (Barón et al., 1995; Rahoutei et al., 1998).

In the leaves of *Abutilon striatum* plants infected with AbMV simultaneously with the symptom development the Fv/Fm slightly decreased compared to the controls while the drop of Fo was considerable (Osmond et al., 1998). In the orange coloured leaf tissue and in the 'green islands' surrounded by veins NPQ had been continuously reduced or stopped. The disability of the chloroplasts to emit/dissipate excitation energy neither by NPQ, pH-dependent quenching nor by thermal irradiation, leads to the loss of more chlorophylls and to the appearance of more severe symptoms.

Peterson and Aylor (1995) studied the spatial distribution of fluorescence in leaves of plants under different biotic stresses *in vivo* by using a new method, the fluorescence imaging. The transformation of chlorosis to mosaic pattern at high light intensity coincided with the loss of fluorescence.

Effect of virus infection on CO_2-fixation and on some biosynthetic processes

In several virus-host interactions the disturbances of CO_2-fixation and of related metabolic ways, or the alteration in ratio of certain products was reported. At the early stage of systemic TMV infection (virus replication) more CO_2 molecules assimilated by the photosynthetic processes (Doke and Hirai, 1970a). As the infection had been progressed this tendency inverted: CO_2-uptake was decreasing in the infected tissues (Doke and Hirai, 1970b; Hunter and Peat, 1973). The same changes as well as the increase of respiration were observed in tobacco leaves infected with tobacco etch *potyvirus* (TEV) (Hopkins and Hampton, 1969b). The enzyme activity of phosphoenol-pyruvate-carboxylase (PEP-carboxylase) increased in the leaves of chinese cabbage infected with TYMV (Bedbrook and Matthews, 1972). In contrast, in tobacco leaves infected with TSWV activity loss of PEP-carboxylase was measured (Mohamed, 1973), and it was explained as the early senescence induced by the virus infection.

In virus infected plants amino acids translocated from the chloroplasts to the cytoplasm, which means that CO_2 built into amino acids instead of free sugars (Magyarosy et al., 1973; Platt et al., 1979). The primary metabolites of the photosynthetic pathway transformed into glucose, amino acids or organic acids, which are not able to translocate from the site of their production (Bedbrook and Matthews, 1973). Sugar and starch accumulation was the result of the disturbed translocation. Therefore metabolites like soluble carbohydrate cannot get from the healthy leaves into the infected ones (Tu, 1978; Mandahar and Garg, 1972). Tomato yellow mosaic *geminivirus* (ToYMV) primarily replicates in the phloem cells of tomato plants causing their pathologic alterations (Leal and Lastra, 1984). This way, virus infection inhibits the translocation of assimilates. But in the case of red clover mottle *comovirus* (RCMV), the distribution is not restricted to the phloem of the host plant. Photosynthetic metabolites transformed into starch, amino acids and organic acids, too (Técsi et al., 1992). Hence starch accumulation was observed in the cells in which virus did not replicate, therefore it could not be a direct or specific effect, but secondary consequences of the upset balance between the sink and source.

Disturbances in the translocation of the photosynthetic assimilates may be related to the synthesis of viral movement protein (MP) (Lucas et al., 1993; Olesinski et al., 1995; Ding et al., 1996, 1998; Helms and Wardlaw, 1977; Herbers et al., 1997). Roberts and Wood (1982) analysed the possible relationship between chlorosis, virus replication

and starch accumulation in two pathogenically different CMV strains. They concluded that the severity of chlorotic symptoms does not depend on the virus content. In the leaves of tobacco infected with the strain inducing strong chlorosis they could not detect more starch than in the control leaves, while in the leaves showing milder symptoms typical pattern of starch accumulation appeared. On the primary leaves of squash cotyledons susceptible to CMV infection chlorotic lesions developed (Cohen and Loebenstein, 1975) while on the mature leaves yellow mosaic pattern occurred systemically, which contained starch grains (Técsi et al., 1994). These starch lesions could be divided into four concentric parts based on their structure. In the middle there were cells showing high photosynthetic activity, with a great amount of starch. The next surrounding zone contained mostly starch-free cells with lower photosynthetic activity, then a transitional zone, and the most outer zone contained cells with higher photosynthetic activity, but without a considerable amount of starch. Starch accumulation was the direct effect of the increased photosynthetic activity rather than the result of the metabolite translocation from the non-infected tissues. In the cells of the lesions the physiological changes were connected to the virus replication, which was supported by the fact that positive and negative sense viral RNA accumulated in the first front line of the infection (Técsi et al., 1996). In the zones of the lesions the activity of the enzymes of the oxidative pentose-phosphate pathway and of the NADP-dependent malate dehydrogenase enzyme grew (Técsi et al., 1996). The spatial distribution of chlorotic symptoms correlated with the increase of glycolysis and respiration, and with the loss of the photosynthetic activity and protein synthesis.

Relationship between the virus and/or its product accumulation and the symptom development

The build up of TMV virions was proven by ultrathin microscopic slices of tobacco chloroplasts (Esau, 1968), or in the chloroplast stroma of Johnsongrass chlorotic stripe *carmovirus* (JoCSV) infected *Sorghum halepense* (Izadpanah et al., 1993). Several authors studied the presence of viruses in the etioplasts of tobacco plants (Pratt, 1969; Honda and Matsui, 1971). Following infection with TMV U5 strain, in the chloroplasts of the susceptible tobacco plants, the size of the observed virions was 1/3 the normal virion length, so the virus-like particles were probably pseudovirions (Shalla et al., 1975). Koiwa et al. (1990) proved the *in situ* presence of TMV in the chloroplasts by electron microscopic slides. It still remains as a matter debate, what role the individual viral constituents like the coat protein (CP), or the "movement proteins" (MP), or other possibly toxic product of the viral genom, or the viral nucleic acids play in the development of different symptoms.

Reinero and Beachy (1986) have described the accumulation of TMV CP and the correlation between the CP concentration and the severity of symptoms. The coat protein was primarily attached to the thylakoid membrane. They proposed that the TMV CP was bound to one of the chloroplast proteins. Reinero and Beachy (1989) compared symptoms caused by TMV strains of different pathogenity, and concluded that the impairment of the electron transport was on the reducing site of PSII. The results of Hodgson et al. (1989) also underlined the hypothesis, that the CP directly inhibited PSII by binding

directly onto it. The 20, 40 and 60 kD proteins which appeared with the onset of infection in the thylakoid membranes have been shown by immunological detection to be the oligomer forms of the coat protein. Thus the impairment of the electron transport was directly induced by the CP. Changes in fluorescence kinetics have also been attributed to the attachment of CP to the thylakoids (Balachandran et al. 1994a), and later to the aggregation of chlorophylls in the light harvesting antenna complex of PSII.

From experiments with coat protein mutant viruses it was evident that even though the virion assembly was inhibited by the modification of the coat protein by gene insertion or deletion, it did not block the replication, or the movement from cell to cell and certain systemisation of the infection (Dawson et al., 1988). The mutant viruses replicated and moved within the cells in the form of free RNA. The protein products derived from abnormal CP genes led to the development of diverse symptoms, but common among them was the appearance of chlorosis. This observation showed that the CP plays an important role in the initiation of the close physiological interaction between the virus and the host plant. In experiments of Banerjee and Zaitlin (1992) with mutant TMV strains and artificially created chimera viruses had shown that a single nucleotide change in the CP gene caused a drastic change in symptoms.

The toxicity of CP was proved by Naderi and Berger (1997) with the use of genetically modified tobacco plants, into which the potato Y *potyvirus* (PVY) CP gene linked with the transit peptide of the small subunit of Rubisco enzyme was built in, so the virus coat protein was transmitted into the chloroplast. The coat protein like products of the CP mutant TMV strains did not accumulate in the chloroplasts (Lindbeck et al., 1991), however still different symptoms were expressed. According to the authors the CP excerted its effect on symptom development outside the chloroplast. Slight and severe symptom producing strains of CMV (where CP did not accumulate in the plastids) were compared by Roberts and Wood (1981) based on the rate of CP accumulation. The strain with strong symptoms produced considerably greater concentration of CP within the cells of the host plant, and there was a direct correlation between the concentration of CP and the development of symptoms.

Viral RNA was detected within the plastids in the case of several virus infections. The presence of these RNAs may indicate the site of viral synthesis, e.g. in TYMV (Ralph et al., 1971), or in BSMV (Lin and Langenberg, 1985). With regards to the synthesis of *potyviruses* it was possible that the replication takes place within the plastids (Mayhew and Ford, 1974; Teakle and Pares, 1977; Gadh and Hari, 1986). During the systemic infection of PVY it was also shown that the CP, the helper component (HC) and the PVY RNA was present in the plastids (Gunasinghe and Berger, 1991). The coat protein was present mainly in the thylakoid fraction rather than in the stroma. It is still an open question whether the gene products of the virus RNA are translated in the chloroplasts or are transported from the cytoplasm. TMV L-RNA devoid of coat protein was detected inside the chloroplasts, while S-RNA was not found (Schoelz and Zaitlin, 1989). The authors therefore doubted the translocation of TMV CP into the plastids, but supposed that the protein is translated by the chloroplast ribosomes from the TMV RNA. An answer to this hypothesis was attempted by Banerjee and Zaitlin (1992), who had

investigated the *in vitro* mobility of the TMV CP into isolated intact chloroplasts. TMV CP quickly mobilised into the chloroplasts, without the preliminary splitting of any transit peptide as was previously observed *in vitro* by Chua and Schmidt (1978) when the smaller subunit of the Rubisco enzyme mobilised into the chloroplast. The process also took place in the absence of ATP, so unlike active transport it does not need an energy source.

The parallel inoculation with (CMV Y) satellite RNA and CMV strain with weak pathogenity produced the modification of symptoms in tobacco (Masuta et al., 1993). The authors considered that the chlorosis induced by the satellite RNA was a result of the inhibition of *de novo* chlorophyll synthesis, because the symptoms were solely expressed in the newly developing leaves.

In the inhibition of photosynthesis and the modification of symptoms not only the coat protein might play a role, but also the different virus MP-s (Helms and Wardlaw, 1977; Ding et al., 1998). TMV MP producing transgenic tobacco plants were made in which the photosynthetic activity was lowered and NPQ was higher. The rate of electron transport decreased. Based on the different chlorophyll fluorescence parameters it seemed likely that the TMV MP did not directly effect any step of the electron transport chain, but caused the specific inhibition of phosphate uptake (Wolf and Millatiner, 2000). The transport of carbon metabolism and CO_2 fixation products into the distal parts of the plant were impaired in these plants (Lucas et al., 1993; Olesinski et al., 1995). The continuous production of TMV MP affected the plasmodesma size dependent permeability (SEL) of the palisade layer in the mesophyll. It was shown that the product transport through the simplast, the distribution of photosynthetic assimilates and the distal transport processes in the phloem were also affected. In the same experiment the build up in the concentration of saccharose, glucose and fructose as a negative feedback blocked the triose phosphate translocator of the chloroplast. Thus stimulated the starch synthesis and storage, which lead to a net reduction in photosynthesis. The TMV MP therefore affected the photosynthetic activity of plants in a complex way.

Herbers et al. (1997) compared the effect of the potato leafroll *polerovirus* (PLRV) MP with the findings of TMV MP to see how general these changes were. The function of the chloroplasts was altered compared to the control, the maximum rate of photosynthesis was lowered. The Rubisco enzyme was not activated by the substrate CO_2, and thus could not function efficiently. The concentration of Rubisco proteins decreased, the concentration of carbohydrates increased, which in turn inhibited photosynthesis. According to Herbers et al. (1997) unlike the TMV MP producing transgenic plant, the PLRV MP blocks photosynthesis by another process. Based on their findings the TMV MP reduced the rate of sugar transport and allocation through the simplast in the mesophyll cells, while the PLRV MP blocks the charging of the apoplast, and the accumulating sugars take part in the extracellular signalling, which leads to the change in the expression of different genes (pathogenesis related /PR/ proteins, genes of photosynthetic proteins).

In BSMV infected barley leaves, with the increase in the concentration of RNA dependent RNA polymerase, the rRNA concentration of chloroplasts decreased (Brakke et al., 1988). This was earlier reported by Hirai and Wildman (1969) in the chloroplasts of TMV infected tobacco, which was explained by the competition between the virus and

the chloroplast for the metabolic products. In the chloroplasts of tobacco leaves infected with TEV the synthesised total protein concentration decreased (Hampton et al., 1966).

Effect of virus infection as a biotic stress factor on photosynthesis

Stress has been described by Osmond et al. (1987) as all parameters that suppress the maximum, potential genomial value of growth and reproduction of a plant. Plants adopt to stress conditions in two ways, either by tolerance or avoidance. In the case of tolerance the plant's reaction to a moderate stress is the upregulation, while against a severe stress the downregulation of metabolic processes.

At the cellular level the damage in the membranes and the fluorescence of chlorophyll built in the thylakoid membranes indicated the post stress conditions. In the early phase of virus infection both the non-photochemical quenching of fluorescence and the predominantly reduced state of Q_A the primary electronacceptor of PSII, indicated the development of symptoms and the rapid chloroplast destruction due to photoinhibitory conditions (Balachandran et al., 1997). The build up of carbohydrates and the brakeup of the equilibrium between synthesis and brakedown probably blocks the gene regulation, which effects the level of chlorophyll-protein complexes and photosynthetic metabolic enzymes.

The plant's reaction to stress was also determined by other abiotic factors, such as the light intensity or the availability of nitrogen (Balachandran et al., 1994b). TMV infection in tobacco plants has multiplied the rate of photoinhibition (which was shown in the high Fo and low Fv/Fm values) especially with low nitrogen concentrations (Balachandran and Osmond, 1994).

Similarly to the abiotic stress processes, stress hormones are also synthesised in virus infected plants. In parallel with the brakedown of chloroplasts in TMV infected tobacco plants, the concentration of abscisic acid (ABA) also rose (Fraser et al., 1986). In the leaves of healthy control plants, in which drought stress was not induced, ABA was located in the chloroplasts, while virus infection enhanced ABA synthesis, changed its location within the cell and blocked the growth (Balázs et al., 1973; Fraser and Whenham, 1989).

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Serological, Pathological and Molecular Characterisation of Hungarian Pepper Mild Mottle *Tobamovirus* (PMMoV) Isolates*

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Last year pepper growers observed symptoms referring to virus-infection in pepper plantations in plastic tunnels. Infected plants showed mosaic symptoms or mottling of the leaves, while on the fruits necrotic spots developed. These symptoms referred to a *tobamovirus* infection. Collected samples were examined by serological and pathological methods, followed by the biological characterisation of the isolates. For serological studies the DAS-ELISA method was used, in which the pathogen was identified as pepper mild mottle *tobamovirus*. During the pathological examination different host-plants have been used including some pepper varieties containing different L genes (L^+–L^4). It was found, that the Hungarian isolates belonged to the $P_{1,2}$ pathotype and were closely related to the Spanish isolate (PMMV-S). PCR-studies proved the presence of the PMMoV $P_{1,2}$ pathotype in Hungary as well.

Keywords: Pepper mild mottle virus, *tobamovirus*, PCR.

It is well known that among pepper diseases the viruses play a major role. Pepper is a natural host of about 45 plant-pathogen viruses (Green and Kim, 1991; Gáborjányi et al., 1997, 1998). These viruses cannot be diagnosed only by the symptoms, as one virus can cause different symptoms, and similar symptoms can derive from different pathogens as well.

Tobamovirus group is an important and separate group of pepper viruses *(Table 1)*, causing serious yield losses especially in the greenhouses or plastic tunnels. Its first member is the tobacco mosaic *tobamovirus* (TMV) mentioned at first more, than 100

Table 1

Pepper-pathogen *tobamovirus*es
(Viruses in bold type are officially recorded by ICTV)

Virus	First description
Tobacco mosaic tobamovirus (TMV)	Mayer, 1886
Tomato mosaic tobamovirus (ToMV)	Clinton, 1909
Tobacco mild green mosaic tobamovirus (TMGMV)	McKinney, 1929
Pepper mild mottle tobamovirus (PMMoV/ PMMV)	McKinney, 1952
Bell pepper mottle virus (BePMV)	Feldman and Oremianer, 1972
Paprika mild mottle tobamovirus (PaMMV)	Rast, 1979
Dulcamara yellow fleck virus (DYFV)	Salamon et al., 1987a,b

*Dedicated to the occasion of the 65th birthday of Professor József Horváth

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years ago (Mayer, 1886; Linhart and Mezey, 1890). After the first description of TMV in Hungary (Szirmai, 1950) many papers dealt with *tobamoviruses*. Horváth (1969) tested different pepper varieties to TMV resistance but did not find resistant varieties among the cultivated sweet and red peppers. The breeders concentrated first on the incorporation of L^1 gene into different varieties (Zatykó, 1982). The first *tobamovirus* in Hungary which overcame the resistance provided by the L^1 gene was isolated in 1980 as ToMV-Ob (Csilléry and Ruskó, 1980). Later Salamon et al. (1987a,b) reported a new pathotype named dulcamara yellow fleck. Burgyán et al. (1978) drew the attention to the heterogeneity of the *tobamoviruses* isolated in Hungary, proving that isolates from infected pepper, tomato, tobacco and *Solanum dulcamara* could be grouped both by their host-plant reactions and serological features. Isolates deriving from pepper represented more independent types. Tóbiás et al. (1982; 1983) dealt especially with the symptomatological and serological behaviour of different *tobamovirus* isolates. They gave a detailed comparison of six pepper-pathogen tobamoviruses including the Hungarian ToMV-Ob isolate and the TMV-SL isolate (later separated as a new virus species, pepper mild mottle virus by Wetter, 1984a,b). Pepper mild mottle *tobamovirus* (PMMoV/PMMV) meant a new and special problem for pepper production. The virus produced slight mottling or no symptoms on the leaves, but caused chlorotic mottling, malformation and reduction in size with occasional necrotic spots on the fruits (Wetter et al., 1984a,b; Avgelis, 1986; Alonso et al., 1989).

The presence of this pathogen in Hungary was supposed earlier. Tóbiás and Csilléry (1983) reported a new TMV pepper strain which systemically infected the peppers containing the L^1 gene. The samples were examined on indicator plants and with ELISA tests. According to these the infected samples gave positive reaction with the TMV-SL antiserum and the isolates represented at least a pathotype of P_1. However it was not further examined, if P_1, or a more aggressive pathotype was present. In a former paper Salamon (1993) mentioned the isolation of PMMV, however, no detailed description was reported about the isolate called PMMV-Nov, except the host-plant tests on the pepper varieties containing different L genes. It was indicated, that PMMV-Nov belonged to the $P_{1,2,3}$ pathotype. Kiss (1996) reported about the infection of PMMV diagnosed by DAS-ELISA in the region of county Csongrád. Our isolates probably represent a new ($P_{1,2}$) pathotype of PMMoV in Hungary. The aim of our work is to characterise these isolates by different methods, to compare them with other *tobamoviruses* and work out a PCR method to identify separately the different *tobamovirus* isolates causing heavy losses in the pepper-plantations.

PMMoV became very important especially in the Mediterranean pepper-growing countries, causing more and more losses (Selassie et al., 1981; Wetter et al., 1984a,b; Avgelis, 1986; Betti et al., 1988; Alonso et al., 1989). Now it seems that it occurs often in the plastic tunnels in Hungary and causes quality degradation of the fruits. Because of the internal seed transmission (Tošić et al., 1980; Tanzi et al., 1989) its presence means a real threat for the pepper production.

As the molecular techniques became general in virology, researchers began to characterise this virus at molecular level too. The nucleotide sequence of its 5' and 3' non-

coding regions was reported in 1989 (Avila-Rincón et al., 1989), followed by the cloning and the complete nucleotide sequence of PMMV-S (Alonso et al, 1991). A procedure involving reverse transcription followed by polymerase chain reaction (RT-PCR) was developed for typing pathotypes of the *tobamoviruses* infecting the L-resistant genotypes of pepper (Tenllado et al., 1994), and to separate the two pathotypes, which cannot be differentiated by serological means. For these reasons PaMMV and PMMV specific primers were used. The discrimination between the two pathotypes was achieved by restriction enzyme analysis of the PCR products. Tenllado et al. (1997) applied the PCR method for the screening of pathotypes in field isolates.

Tobamovirus *pathotypes*

Differentiation of pepper-pathogen tobamoviruses is based on the resistance of wild *Capsicum* species. Resistance in the *Capsicum* species is provided by five, dominantly inherited L genes (L^+, L^1, L^2, L^3, L^4), located on the same locus and localising the infection in an incomplete way. In contrast to it the virus has four pathotypes (P_0, P_1, $P_{1,2}$, $P_{1,2,3}$). The different compatible or incompatible pepper-virus relationships are summarised in *Table 2*. The pepper containing the L^+ gene is susceptible to the infection of any pathotypes. The resistance gene L^1 provides resistance against the P_0 pathotype, the L^2 gene against the P^1 pathotype, etc. If pepper varieties of a certain resistance level are grown for a long period of time, one could observe the tendency, that the virus overcomes the resistance, and a more aggressive strain appears.

Table 2

Relationship of pepper varieties with different resistance level and *tobamoviruses* of different pathotypes (according to Rast, 1979, 1988; Boukema et al., 1980; Tóbiás et al., 1982; and Boukema, 1984)

Resistance genes	Pathotypes			
	P_0	P_1	$P_{1,2}$	$P_{1,2,3}$
L^+	S	S	S	S
L^1	R	S	S	S
L^2	R	R	S	S
L^3	R	R	R	S
L^4	R	R	R	R

S = susceptibility, R = resistance.

According to Betti et al. (1986, 1988) the reason for increasing crop losses was due to the higher level of pathogenicity of the *tobamoviruses*. If one TMV isolate (P_0) was maintained on a L^+/L^1 heterozygote plant within controlled circumstances by repeated passage, the virus overcame the L^1 resistance. Similarly, more aggressive isolate developed when the virus strain was prolonged on more resistant (L^1 or L^2) plants. The increase of pathogenicity on pepper was accompanied by the decreased pathogenicity to tobacco. The virus strain was gradually adapted to the new host and collaterally was losing its

relationship to the original host-plant. On the basis of these facts it could be theoretically supposed, that such pathological change ensued under the Hungarian growing conditions too. Our pepper production is more and more exposed to the appearance of new isolates with higher pathogenicity. The reason of increasing pathogenicity was the preference of resistant varieties, with no hypersensitive response. In these cases the pathogen could replicate at a lower level, but could adapt gradually. If more pathogenic strains became dominant in the population it could be a starting-point for heavy epidemics.

Materials and Methods

In the autumn of 1999 eighteen samples were collected from the infected peppers grown under plastic tunnels in the region of "Jászság" (Middle Hungary). Plants showed mild mosaic leaf symptoms or were symptomless, but necrotic spots developed on the fruits before ripening. First isolates were tested by serological methods followed by host-plant tests and molecular characterisation.

For serological examination the DAS-ELISA method was used according to Clark and Adams (1977), following the instructions of the producers of the ELISA-kit. After collecting the samples they were immediately deep-frozen, and a few days later homogenised in extraction buffer (1:2.5 g/ml), and tested with polyclonal antisera (Loewe Phytodiagnostica). Tests were made to the following viruses: tobacco mosaic *tobamovirus* (TMV), tomato mosaic *tobamovirus* (ToMV), pepper mild mottle *tobamovirus* (PMMoV), potato X *potexvirus* (PVX), potato Y *potyvirus* (PVY), alfalfa mosaic *alfamovirus* (AMV), and cucumber mosaic *cucumovirus* (CMV).

Pepper species with different resistance level and 3 different tobacco species were used for the host-plant tests *(Table 3)*. The resistance level of pepper varieties was the following: L^+ (Albaregia, Synt. Cecei), L^1 (D. Cecei, Fehérözön), L^2 (Greygo), L^3 (Bölény, Rapires), L^4 (Hímes). The deep-frozen samples were homogenised in phosphate buffer (0.1 M) according to Sörensen (pH 7.0; 1:5 w/v) in a mortar with pestle, and the leaves of the host-plants were inoculated mechanically. On peppers the cotyledons, on tobaccoes the lower 3–4 leaves were inoculated. To improve the efficiency of the inoculation silicium carbide (Carborundum 500 mesh) dust was added. The plants were kept in glasshouse (18–25 °C; 160 $mEm^{-2}s^{-1}$ additional lighting for 8 hours a day; relative humidity 60%). Local symptoms were checked after 4–7 days, and systemic symptoms after 3–4 weeks.

For the molecular studies a universal primer pair was designed to recognise the most *tobamoviruses*. Sequences of the *tobamoviruses* were taken from the Genbank of EMBL Data Library, DNA Data Bank of Japan. The most important viruses under the Hungarian conditions were selected because of the great amount of the *tobamovirus* sequences. We tried to find a section, what was quite the same in every (or at least in the most important) *tobamoviruses,* where the universal primer pair was designed *(Fig. 1)*. The 5' primer was constructed in front of the coat protein (CP) gene, it was degenerated and virussense. The 3' primer was also degenerated, reverse complementary and situated in the

Table 3

Symptoms of indicator plants inoculated with the samples identified as PMMoV by DAS-ELISA

Isolates*	CPSAN** L^+	CPSAN L^1	CPSAN L^2	CPSAN L^3	CPSAN L^4	NIOTA-XNC	NIOTA-SAM	NIORU
Ca 2	–/Mot***	–/Mot	–/–	Ab/–	Ab/–	LLe/–	–/s	LLe/–
Ca 2a	–/Mot	–/Mot	–/–	Ab/N	Ab/–	LLe/–	–/M	LLe/–
Ca 4a (mixed with PVY)	–/Mot	–/Mot	–/–	Ab/–	Ab/–	LLe/ VN,N, M, Ch	–/s	LLe/–
Ca 5a	–/Mot	–/Mot	–/–	Ab/–	Ab/–	LLe/–	–/N, VN	LLe/–
Ca 5b (mixed with CMV)	–/–	–/Mot	–/–	Ab/–	Ab/–	LLe/–	–/s	LLe/–
Ca 6	–/–	–/Mot	–/–	Ab/–	Ab/–	LLe/–	–/s	LLe,N/–
Ca 7	–/Mot	–/Mot	–/–	Ab/–	Ab/–	LLe/–	–/s	LLe/–
Ca 8 (mixed with PVY)	–/Mot	–/Mot	–/–	Ab/–	Ab/–	LLe/ M, VN	Ch/ VN, N	LLe,Ch /–
Ca 10 (mixed with ToMV)	–/Mot	–/Mot	–/–	Ab/–	Ab/–	LLe/–	–/s	LLe/–

Key:

* Ca 2, Ca 2a, Ca 4a, Ca 5a, Ca 5b, Ca 6, Ca 7, Ca 8, Ca 10 = pepper isolates,
PVY = potato Y virus, CMV = cucumber mosaic virus, ToMV = tomato mosaic virus.

** Abbreviations of host-plants are used according to Horváth (1993). CPSAN = *Capsicum annuum,* NIOTA-XNC = *Nicotiana tabacum* cv. Xanthi-*nc,* NIOTA-SAM = *Nicotiana tabacum* cv. Samsun, NIORU = *Nicotiana rustica.*

*** Abbreviations of local symptoms can be seen in the numerator, while systemic symptoms are indicated in the denominator. Mot = mottling; – = no symptoms; Ab = abscission; L = local; Le = lesion; Ch = chlorosis; VN = vein necrosis; N = necrosis; M = mosaic; s = symptomless infection (detected with back inoculation to *Nicotiana tabacum* cv. *Xanthi*-nc).

3' non-coding region. The universal primer pair was successfully tried out with own and authentic isolates. The authentic isolates originated from the stock-collection of the Plant Protection Institute. The PCR was carried out according to the following description:

Nicotiana tabacum cv. Samsun plants were mechanically infected as mentioned above with the own and authentic isolates. Samples of 0.1 g were taken about 3–4 weeks after the inoculation from the systemically infected leaves. Total RNA was extracted from plant tissues, using the following method: samples were homogenised in 600 µl extraction buffer (pH 7.6 containing 5% TRIS (1.0 M), 2% NaCl (5.0 M), 0.2% EDTA (0.5 M), 5% SDS (10%), 0.3% mercaptho-ethanol in 100 ml distilled water) adding 600 µl phenol, then centrifuged for 5 minutes (15000 rpm). 300 µl phenol and 300 µl chloroform was added to the supernatant and centrifuged for 5 minutes. After adding 600 µl chloroform to the supernatant the centrifugation was repeated, and adding 1 µl absolute alcohol to the supernatant was followed by centrifugation (10 min) again. The total RNA was precipitated and washed with ethanol (70%), then after drying suspended in sterile distilled water (60 µl).

a. The 5' end of the primer pair

TMV-Ob TATGAGTCAG GACGGTAGCT CAAGC**GAGAT ATCATCCGAT TCGTTTATTT**
TMV-U1 .ATCGATGAT GATTCGGAGG CTACT**GTCGC CGAATCGGAT TCGT. . .TTT**
PMMoV .ATTGGGCAG AACTCGGAGT CATCG**GACGC CGAGTCTTCT TCGT. . .TTT**
Univers. 5' GACGC GGAGTCGGAT TCGT ATT
T C T T T

↓ CP start

TMV-Ob **AATCATG**CCT TATACTGTTA CGTCTCCAAG CCAACTAGTT TATTTTGGTT
TMV-U1 **AAATATG**TCT TACAGTATCA CTACTCCATC TCAGTTCGTG TTCTTGTCAT
PMMoV **AACTATG**GCT TACACAGTTT CCAGTGCCAA TCAATTAGTG TATTTAGGTT
Univers. AAATATG 3'

b. The 3' end of the primer pair

TMV-Ob TACGACTATA AAAATAAGAA GGGGTTCGAA TCCCCCCCGT **ACCCCGGGTA**
TMV-U1 CAGGCACGT. AATAAAGCGA GGGGTTCGAA TCCCCCCGTT **ACCCCCGGTA**
PMMoV AACGACGTAA TTATTTTTCA GGGGTTCGAA TCCCCCCCGA
ACCGCGGGTA

TMV-Ob **GGGGCCCA**
TMV-U1 **GGGGCCCA**
PMMoV **GCGGCCCA**

Universal: 5' TGGGCC (GC) CTACC (GC) G (GC) GG 3'

Fig. 1. The universal primer pair

a. The TMV-Ob specific primer.

TMV-Ob ATAGTATA**GG AGTTTCCAAA CCTGTCGG**TT TGGAAAGAAA TAAAGTAAGG
TMV-U1 AAAATAGTAG TAATGATCGG TCAGTGCCGA ACAAGAACTA TAGAAATGTT
PMMoV AGAGAAATGA TAATAAGGGT T. . . . . TGA ATAAGGAAGG GAAGCTGTTT

b. The TMV-U1 specific primer.

TMV-Ob GGGAATCAGT TTCAAACACA GAATGCGAGA ACTACTGTAC AACAGCAGTT
TMV-U1 GGAAATCAGT TTCAAACACA ACAAGCTCGA ACT**GTCGTTC AAAGACAATT**
PMMoV GGCAATCAGT TTCAAACACA ACAGGCTAGA ACTACGGTTC AACAGCAGTT

TMV-Ob CAGTGATCTG TTTAAGACTG TTCCCACTAG GACCAATAGG TTCAATGATG
TMV-U1 **CAGTGAG**GTG TGGAAACCTT CACCACAAGT AACTGTTAGG TTCCCTGACA
PMMoV CTCTGATGTG TGGAAGACTA TTCCGACCGC TACAGTTAGA TTTCCTGCTA

c. The PMMoV specific primer.

TMV-Ob CTGTTTGGGC GGATCCGATT ACATTTATCG ATCTTTGTAC CGTGGCCTTG
TMV-U1 CAGCGTGGGC CGACCCAATA GAGTTAATTA ATTTATGTAC TAATGCCTTA
PMMoV CTGTATGGGC TGAT**CCATTA GAGTTACAAA ATCTGTGTAC TTC**GGCGTTA
T

Fig. 2. The virus-specific primers

For cDNA synthesis, 1 µl 3' primer (100 pmol/µl cc.) and 3.75 µl distilled water was added, and denaturised at 65 °C for 5 minutes. Afterwards 2 µl reverse-transcriptase buffer (5x), 0.25 µl reverse-transcriptase enzyme and 1 µl dNTP mix (5mM) was added to the mixture, and kept at 42 °C for one hour. In the final reaction tube 2 µl of the former synthetised DNA, 5 µl PCR buffer (10x), 3 µl $MgCl_2$ (25 mM), 2 µl dNTP mix (5mM), 1–1 µl of both primers, and 36 µl distilled water was mixed and 0.5 µl Taq polymerase enzyme was added. The enzymes and reagents were produced by Fermentas. The amplifications were carried out using the following programme: 94 °C denaturation for 3 minutes, 35 cycles (one cycle was consisted of 94 °C for 15 seconds, 60 °C annealing for 30 seconds, 72 °C extension for 2 minutes) and 10 minutes final extension at 72 °C. The PCR-products were analysed in agarose gel (1.2%) in the presence of 0.001% ethidium bromide, using 45 mM Tris-borate pH 8.0 1 mM EDTA (TBE) as electrophoresis buffer (Sambrook et al., 1989) at 120 mA.

Specific primers were designed to the most important *tobamoviruses (Fig. 2).* These primers were created to a section, where the other *tobamoviruses* were different. (The 3' primer of the universal primer pair was used as the other half of these specific primer pairs.) The TMV-Ob specific primer was situated in front of the CP gene, while the TMV-U1 specific primer and the PMMoV specific primer were located in the CP gene. During the designing of the specific primers we had the aim to construct primers recognising different-sized PCR products *(Fig. 3).* Later we would like to use them in a multiplex-PCR, in which the *tobamoviruses* could be identified according to the size of the PCR products. The specific primers were also tested with more isolates following the description detailed above.

Universal primer pair:

in case of PMMoV: 699 bp
in case of TMV-U1: 709 bp
in case of TMV-Ob: 782 bp

Specific primers:

in case of PMMoV: 613 bp
in case of TMV-U1: 555 bp
in case of TMV-Ob: 899 bp

Fig. 3. The estimated length of the sections recognised by the primers

Results

In ELISA tests 9 samples were infected by PMMoV, from which 4 were infected with other viruses too. Altogether 3 samples were infected by CMV, 4 by PVY, and 1 by ToMV. In some cases complex-infection was detected, but no pathogen was found in 5 samples.

Tests were continued with samples containing the PMMoV. Symptoms were summarised in *Table 3.* Most isolates caused systemic mottling on the pepper varieties containing the L^+ or L^1 gene, while no mottling could be detected on the pepper varieties of L^2 resistance level. Pepper varieties containing the L^3 or L^4 gene were resistant, responded to the infection with leaf-abscission (hypersensitive response). On *Nicotiana tabacum* cv. Xanthi-*nc* plants local lesions were observed in every case, no systemic infection occurred except in the case of mixed-infection, where the other viruses of the complex (CMV, PVY) could be responsible for the systemic symptoms. On the *Nicotiana rustica* plants local lesions developed, but no symptoms were observed at all on *Nicotiana tabacum* cv. Samsun.

A lot of examinations have been carried out with the universal primer pair to find the best parameters and to prove that this primer pair was able to recognise the *tobamovirus* isolates. *Fig. 4* demonstrates the results of a PCR examination where different authentic and own isolates were recognised by the universal primer pair. On the basis of PCR products every isolate proved to be a *tobamovirus.* On the other hand, the universal primer pair was able to recognise every *tobamovirus* isolates.

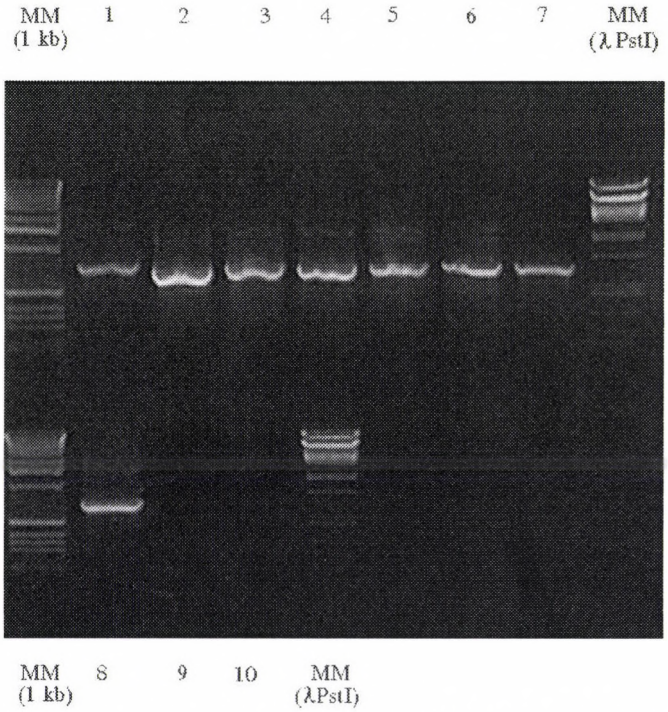


Fig. 4. PCR examination of the Ca 2 isolate using the universal and the PMMoV virus-specific primers. PCR products were analysed in agarose gel (1.2%). Line 1: isolate Ca 2 with the universal primer pair (699 bp); lines 2–3: isolate Ca 2 with the PMMoV-specific primer (in repetition, 613 bp); line 4: TMV-U1 isolate with the TMV-U1 specific primer (as comparison, 555 bp); MMa: size-marker (1 kb); MMb: size-marker (λPstI)

Fig. 5 shows a PCR test. One of our isolates, called Ca 2 (identified as PMMoV with ELISA and test plants) was tested with the universal and PMMoV specific primers. PCR products were detected by using the universal primer pair, as well as with the PMMoV specific primer. It confirmed the former results, that the isolate Ca 2 was PMMoV. Naturally, the isolate Ca 2 was tested with the TMV-U1 and TMV-Ob primer as well, but in these cases – as it was expected – no PCR products were detected.

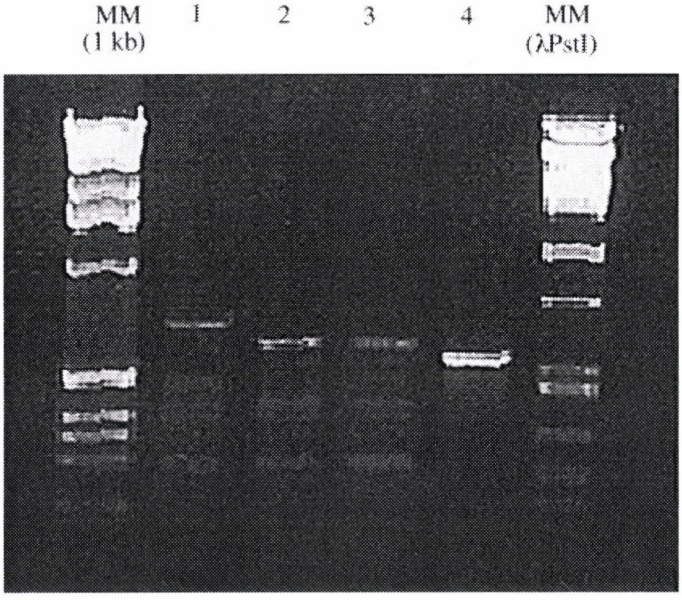


Fig. 5. PCR examination of *tobamovirus* isolates by using the universal primer pair. Lines 1–5: authentic *tobamovirus* isolates, line 1: SL 5x; line 2: SL; line 3: TMV-U1; line 4: ToMV-Ob; line 5: ToMV D/H; lines 6–8: own isolates; line 6: And; line 7: Ca 6; line 8: Ca 5a; line 9: healthy plant; line 10: control; MMa: size-marker (1 kb); MMb: size-marker (λPstI)

Discussion

PMMoV (*versus* PMMV) was first described by McKinney in 1952. Former synonym names of the virus were: Samsun latent strain of TMV, pepper mosaic virus, *Capsicum* mosaic virus, pepper unusual strain, isolate P 8, isolate P 14, SL-TMV.

Serological tests carried out with the specific antiserum of different *tobamoviruses* confirmed our hypothesis that a *tobamovirus* was present. Within the *tobamovirus* group the pathogen was identified as pepper mild mottle *tobamovirus* (PMMoV).

Two important PMMoV/PMMV isolates were separated so far, which differ in their pathotype (Boukema et al., 1980; García-Luque et al., 1990, 1993). The first one

was the Spanish isolate (PMMV-S) a pathotype of $P_{1,2}$, and the other was the Italian isolate (PMMV-I) with higher level of pathogenicity ($P_{1,2,3}$). Spanish isolate caused resistance only on the peppers containing the L^3 or L^4 gene, while the peppers of L^1 and L^2 resistance level were susceptible to its infection. The infection was symptomless on the *Nicotiana tabacum* cv. Samsun, while local lesions could be detected on the *Nicotiana tabacum* cv. Xanthi-*nc* and *Nicotiana rustica* plants. Similar symptoms could be observed in the case of the Italian isolate with the exception that only the L^4 pepper was resistant to its infection.

Comparing our results to the literature data it was concluded, that our samples were infected by PMMoV. The symptoms developed on the tobacco plants were more or less coincide with the data in the literature (Tóbiás et al., 1983), while the symptoms on pepper plants decided, that from the two PMMoV isolates in Hungary the less aggressive $P_{1,2}$ pathotype was found. According to the literature this pathotype caused systemic mottling on the L^2 peppers, but we did not observe symptoms at all. It was probably due to the fact, that this virus – as it is shown in its name – often causes very weak or no symptoms. No leaf-abscission was observed as in case of the resistant varieties, therefore the peppers of resistance level L^2 are susceptible to its infection.

Examination of the PMMoV isolates in nucleic acid level confirmed the results of our former serological and pathological studies. Tests with the universal primer pair demonstrated that the isolates belonged to the *tobamovirus* group, while tests carried out with virus-specific primers proved that the isolates were identical with PMMoV. The PCR-based method was efficient to identify the Hungarian *tobamovirus* isolates rapidly and easily using universal and virus-specific primers.

Acknowledgement

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Relationship between Resistance to Barley Yellow Dwarf Virus in Spring Barley and Aphid Feeding Behaviour

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The feeding period of the grain aphid, *Sitobion avenae,* an important vector of barley yellow dwarf virus (BYDV), was studied in six varieties of spring barley (*Hordeum vulgare* L.) in an attempt to explain the resistance mechanisms in some barley varieties to BYDV. There was no significant difference in aphid feeding period between the resistant and susceptible varieties. The mechanisms underlying BYDV resistance do not seem to involve factors related to alterations in the feeding period. Suggestions for future studies are highlighted.

Keywords: Barley yellow dwarf virus; feeding period; *Sitobion avenae;* susceptible; tolerance.

Barley yellow dwarf virus (BYDV) is found world-wide, it has been identified in at least one country in every continent. It is the most widely distributed of all the cereal viruses (Rochow, 1970; Lister and Ranieri, 1995). Economic losses have been associated with BYDV in the U.S.A. (Oswald and Houston, 1953), U.K. (A'Brook, 1974), and New Zealand (Smith, 1993).

At least 25 aphid species have been reported as vectors of BYDV (Plumb, 1977). In the U.K., the grain aphid *Sitobion avenae,* the rose grain aphid *Metopolophium dirhodum* and the bird cherry aphid *Rhopalosiphum padi* are the most important BYDV vectors (A'Brook and Dewar, 1980). In spring-sown cereals *S. avenae* is considered to be the principal source of primary infection to the crop (Plumb, 1977). Little is known regarding aphid feeding as a component of BYDV resistance despite the fact that resistance in cereals is related to aphid activity (Ullman et al., 1988).

It is thought that early sowing reduces aphid colonisation prior to stem elongation and hence decreases damage from virus but even this may not be adequate after very mild winters when aphids begin to fly into the crop early in the season. Application of insecticides to control BYDV in spring-sown cereals has been attempted with little success in the U.K. (Irwin and Thresh, 1990). In order to assess the potential of some fortuitously discovered "resistant"/ "tolerant" varieties, it is essential to understand the mechanism by which such resistance or tolerance is conferred. This study sought to determine whether or not inoculation access feeding period (IAFP), a component of feeding behaviour, is responsible for the apparent BYDV resistance in three varieties of spring barley.

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Materials and Methods

The aphid cultures

Virus-free cultures of *S. avenae* were maintained on barley (cv. Magie) at 18 hour light and 6 hour dark cycle (L 18:D6) at 18 °C. The culture plants were changed weekly and population of aphids was reduced at each plant change in order to avoid overcrowding. Viruliferous cultures of *S. avenae* were started with aphids from the virus-free cultures but were kept in a separate place to avoid contamination of the virus free stocks. Aphids were removed from the culture and placed on oat plants (cv. Dula) infected with the MAV-like isolate of BYDV and kept in a cage in the laboratory at approximately 21 °C. These aphids were removed after 72 hours virus acquisition access period and kept in glass tubes for an overnight starvation period.

The plant materials

Test plant used in this study were three BYDV "resistant" (Cooper, R1; Optic, R2; and Amber, R3) and three "susceptible" (Alexis, S1; Derkado, S2 and Triumph, S3) varieties of spring barley. Oat (cv. Dula) was used as a control since it is thought to be the most susceptible cereal to BYDV. Ten seeds of each variety were planted in peat jiffy pots placed in plastic trays. One set was used for the four feeding periods (IAFP).

Inoculation of plants with BYDV

The IAFP used were 6, 12, 24 and 48 hours (Lowles et al., 1996). *Sitobion avenae* specific isolate of BYDV (the MAV-like isolate) was used in all the experiments. One week old test plants were used since this stage is the most susceptible to the disease and the potential for yield loss is very high (Doodson and Saunders, 1970). The test plants were inoculated with the virus by confining one winged viruliferous *S. avenae* per plant for the required length of time. The aphids were contained in a 2.5 cm diameter cellulose acetate tube with a netting top which was pushed into the soil around the plant. The aphids were put at the base of the plants by means of a pair of forceps clipped to one of the wings so as to allow the aphids to freely find their own feeding site on the plants. After each IAFP, each aphid was removed and any nymphs killed simply by crushing them to death. The ten plants for each variety/time combination were transferred to a separate glasshouse for spraying to kill any nymphs missed. Pirimicarb (a contact insecticide with some systemic activity) at 0.5 g a. i. litre^{-1} was used. The plants were then removed to the glasshouse and watered regularly until they were ready for the serological test. In the glasshouse the mean temperature was 21 °C, the maximum 26 °C and the minimum 16 °C during the study. In total, 6 replicate runs of the experiment were carried out and a completely randomised block design was used. Two hundred and eighty aphids and two hundred and eighty plants were used per run (i.e. one aphid per plant). The indirect double antibody sandwich ELISA (Lister and Rochow, 1979) was used in testing the plants for the presence of BYDV 21 days after inoculation was carried out.

Data analysis

Data were analysed and run on the Rothamsted GENSTAT computer programme. Logistic regression model via the generalised linear model (GLM) was fitted to proportion of plants infected. The effect of time was split into a linear component to see whether proportions changed linearly with time or whether any other relationship could be observed. Overall differences between the six replicate runs were accounted for before fitting treatment terms. Treatment differences were not assessed using conventional chi-square statistics since the Residual Mean Deviance (RMD) was greater than 1. Hence the mean deviances (chi-square) were scaled to RMD to produce approximate F ratios.

Results

ELISA test results are summarised in *Table 1*. There was no significant relationship between;

a) The proportion of plants with virus and inoculation access feeding period (6hr, 12hr, 24hr and 48hr). **b)** Oats, all the "susceptible" (Alexis, S1; Derkado, S2; and Triumph, S3) combined together as a single entry and all the "resistant" varieties (Cooper, R1; Optic, R2; and Amber, R3) also combined together as a single entry. **c)** Oats, all "susceptible" varieties (Alexis, S1; Derkado, S2; and Triumph, S3) as individual entries and all the "resistant" varieties (Cooper, R1; Optic, R2; and Amber, R3) combined together as a single entry. **d)** Oats, all the "susceptible" (Alexis, S1; Derkado, S2; and Triumph, S3) and "resistant" (Cooper, R1; Optic, R2; and Amber, R3) varieties as individual entries.

Table 1

Accumulated analysis of deviance: proportion of infected plants and feeding periods as shown by ELISA

change	df	deviance	mean deviance	deviance ratio
+ TIME	3	10.311	3.437	1.26^{NS}
+ F1	2	6.870	3.435	1.26^{NS}
+ F1. F2	2	4.58	2.293	0.84^{NS}
+ F1. F2. F3	2	0.298	0.149	0.05^{NS}
+ TIME . F1	6	4.523	0.754	0.28^{NS}
+ TIME . F1. F2	6	3.919	0.653	0.24^{NS}
+ TIME . F1. F2. F3	6	3.727	0.621	0.23^{NS}
Residual	140	381.539	2.725	
Total	167	415.773	2.490	

NS = Not statistically significant
TIME = Inoculation access feeding periods (6hr, 12hr, 24hr and 48hr)
F1= Oats **Vs** S1 + S2 + S3 **Vs** R1 + R2 + R3
F2 = Oats **Vs** S1 **Vs** S2 **Vs** S3 **Vs** R1 + R2 + R3
F3 = Oats **Vs** S1 **Vs** S2 **Vs** S3 **Vs** R1 **Vs** R2 **Vs** R3

Also, no significant relationship was also observed between;

a) Inoculation access feeding period, Oats, all the "susceptible" (Alexis, S1; Derkado, S2; and Triumph, S3) combined together as a single entry and all the "resistant" varieties (Cooper, R1; Optic, R2; and Amber, R3) also combined together as a single entry *(Fig. 1).* **b)** Inoculation access feeding period, Oats, all "susceptible" varieties (Alexis, S1; Derkado, S2; and Triumph, S3) as individual entries and all the "resistant" varieties (Cooper, R1; Optic, R2; and Amber, R3) combined together as a single entry. **c)** Inoculation access feeding period, Oats, all the "susceptible" (Alexis, S1; Derkado, S2; and Triumph, S3) and "resistant" (Cooper, R1; Optic, R2; and Amber, R3) varieties as individual entries.

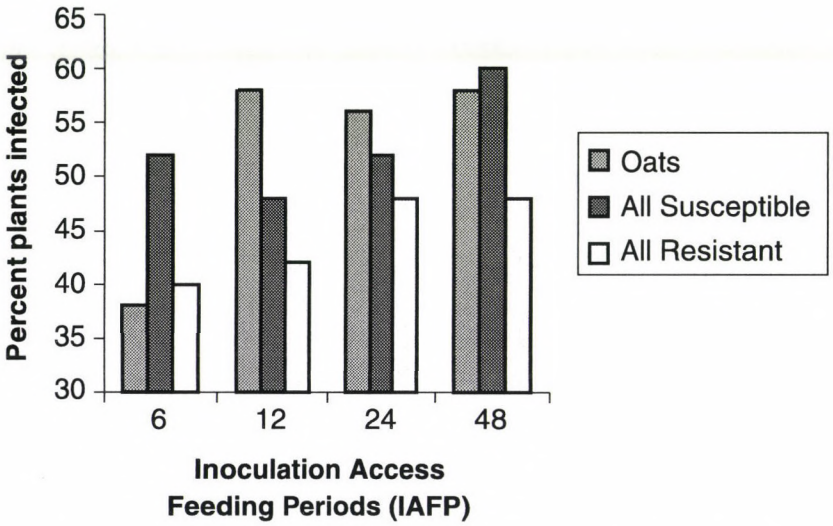


Fig. 1. Proportion of plants infected versus inoculation access feeding periods (Time) in hrs

Discussion

Reports by Haniotakis and Lange (1974) and Tjallingi (1994) showed that plant resistance to aphids and possibly BYDV transmission is associated with an increase in the time required by aphids to penetrate the phloem. The results in this experiment did not agree with such reports *(Table 1).* It was suggested that there could be resistance against aphids in some grasses making them difficult to be infected with BYDV. The results obtained in this study did not show difficulty in getting any of the varieties infected with the virus. ELISA test results showed that the virus infects all the varieties studied and in general, when Oats, all "susceptible" varieties and all "resistant" varieties were compared, it was observed that the "resistant" varieties had lower but not significantly different infection levels in comparison to the others at all the inoculation access

feeding periods used *(Fig. 1)*. This indicates that host effect on feeding period cannot adequately explain the mechanism behind such resistance. Tolerance seems unlikely because even with the apparently resistant varieties, some plants showed symptoms and these symptoms were as strong as those in the "susceptible" varieties. Also ELISA titre values indicated that there was no possibility of tolerance in these fortuitously discovered spring sown "resistant" varieties of spring barley. In autumn sown barley, tolerance is reported to be governed by a single major gene of Ethiopian origin designated as Yd2 gene (Rassmusson and Schaller, 1959).

Conclusion

The mechanism governing BYDV resistance in these varieties of spring barley studied is not well explained by a host plant effect on feeding period. Work is continuing at Rothamsted on the other aspects of aphid feeding behaviour such as rate of probing, depth of probing etc. ELISA results indicated the unlikeliness of tolerance.

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Characterization of Yugoslavian *Erwinia amylovora* Isolates Originating from Apple Trees Comparing with Other Strains of the Pathogen

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Pathogenicity, cultural and biochemical characteristics of the *Erwinia amylovora* strins of various origin were ivestigated. Six of them originated from apple (Yugoslavia and Canada), and four from pear and quince trees (England and Yugoslavia). No differences were observed in any property among the strains investigated.

Keywords: *E. amylovora*, apple strains, cultural characteristics, pathogenicity.

From 1989 when the *E. amylovora* was detected for the first time in Yugoslavia up to 1995 the pathogen prevailed on pear and quince as well as on mespil trees (Arsenijević et al., 1991; Panić and Arsenijević, 1993). Its occurrence in apple orchards was rear of the weak intensity (Panić and Arsenijević, 1993, 1996).

However, during 1995 and 1996 the first fire blight symptoms suddenly outbreaked and spread in numerous district and orchards where apple cultivars were grown (Arsenijević et al., 1996). So behaviour of the pathogen and the strong infection of the apple trees six years after the first outbreak and spread of the fire blight disease in pear and quince orchards, we thought, may be explained by the heterogeneous population of the *E. amylovora* bacterium which probably exists in Yugoslavia.

Therefore, it was a main reason to investigate pathogenicity and bacteriological characteristics of the *E. amylovora* pome strains isolated in Yugoslavia and abroad (England and Canada) from apple, pear and quince trees.

Materials and Methods

The strains used for investigation and their preliminary identification

From the diseased apple trees many strains of *E. amylovora* were isolated in Yugoslavia by usual procedure (Lelliott and Stead, 1987; Klement et al., 1990). Five of them (Ea-4, Ea-72, Ea-75, Ea-83, Ea-95) originated from different localities and apple cultivars were chosen for further study. As the check strains five *E. amylovora* isolates were used: two from England (NCPPB 595 isolated from pear by Dr. J. E. Crosse in 1958 and 5378 from quince isolated in 1983; both strains were received from Dr. Steve Roberts, HRI, Wellesbourne), one from Canada (E81004A received from Dr. W. G. Bonn,

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Harrow, Ontario isolated in 1981 from apple) and two from Yugoslavia (Ea-205 and Ea-311 isolated in 1996 from pear and quince, respectively). All the strains used were stored on NAG (Nutrient agar + 2% glycerol) slopes at 4 °C.

Preliminary identification of the strains studied was carried out using the following tests: Gram differentiation, tobacco hypersensitivity, fluorescence on King's medium B, slide agglutination test and artificial inoculation of immature pear fruits. The immature pear fruits (cv. Williams) were inoculated by pricking using bacterial suspension 10^7 cfu/ml. Slide agglutination test was also performed using conventional procedure and antisera prepared by authentic *E. amylovora* apple strain (1430), obtained from Dr. J. P. Paulin (Station de Pathologie Végétale, Angers, Beaucouzé, France). All tests were carried out according to Klement et al. (1990).

Plant inoculation and cultural and biochemical properties

In order to check the pathogenicity of the strains investigated herbaceous and woody plants were used. The leaves of black night-shade (*Solanum nigrum* L. Mill.), Pelargonium, simpson weed (*Datura stramonium* L.) and tomato were inoculated according to standard procedure (Klement, 1963).

In pathogenicity tests immature apple fruits, quince, plum, sweet cherry, *Sorbus,* Japanese apple fruits were used. Apple, pear, quince, firethorn raspberry and blackberry shoots were also studied. For both, shoots and immature fruits inoculation the bacterial suspension (10^7 cfu/ml) were introduced by pricking into the plant tissues (Klement et al., 1990). The inoculation fruits and shoots were kept in a moisture chambers for three days, and then kept at laboratory conditions.

Growth characteristics of the *E. amylovora* strains were examined on sucrose nutrient agar (SNA) and on Crosse-Goodman (CG) selective medium (Lelliott and Stead, 1987). Tolerances to 5% and 7% NaCl were investigated according to Fahy and Persley (1983; loc cit. Arsenijević, 1997).

Glucose metabolism, oxidase and catalase activity, starch and Tween 80 hydrolysis, indole, H_2S and NH_3 production, methyl red and VP tests were also studied (Lelliott and Stead, 1987; Klement et al., 1990).

Carbohydrates utilization and alkali production from organic acid were analysed according to Klement et al. (1990) and Kim et al. (1996) *(Table 1).* The solid media were streaked using 24-hr-old bacteria cultured on nutrient agar. All tests were carried out at 26 °C.

Results

Preliminary identification characteristics and pathogenicity

No differences among the investigated strains were noticed. All *E. amylovora* strains were asporogenous, gram-negative and rod-shaped. On King's medium B no green fluorescent pigment appeared. Tobacco hypersensitivity and immature pear fruits tests were positive as well as the slide agglutination test.

Table 1

Biochemical and physiological properties of the *E. amylovora* strains investigated

Test	Apple strains (Ea-4, Ea-72, Ea-75, Ea-83, Ea-95, E 81004A)	Pear strains (NCPPB 595, Ea-205)	Quince strains (5378, Ea-311)
O/F test on glucose			
aerobic	+	+	+
anaerobic	+	+	+
Hydrolysis of:			
starch	–	–	–
tween 80	–	–	–
Production of:			
indole	–	–	–
NH_3	–	–	–
H_2S	–	–	–
VP test	+	+	+
Methyl red	–	–	–
Activity of:			
oxidase	–	–	–
catalase	+	+	+
Utilization of:			
glucose	+	+	+
fructose	+	+	+
sucrose	+	+	+
xylose	+	+	+
lactose	–	–	–
mannitol	+	+	+
sorbitol	+	+	+
inositol	+	+	+
glycerol	+	+	+
acetate	+	+	+
citrate	+	+	+
D (–) tartrate	+	+	+
cis – aconitic acid	+	+	+

+ positive; – negative result

All investigated *E. amylovora* strains incited HR on Pelargonium, black nightshade, simson weed and tomato leaves. On pome and stone immature fruits artificially inoculated, they caused tissue necrosis and bacterial oozing formed on pear (cv. William's), apple (cv. Gloster), quince (cv. Leskovacka), plum (cv. Stanley), sweet cherry (cv. Sue) and *Sorbus* (domestic spontaneous population), but not on Japanese apple fruits.

Strong tissue necrosis followed by fire blight symptoms appeared on inoculated shoots of pear (cv. William's), apple (cv. Jonathan), quince (cv. Leskovacka) and firethorn (*Pyracantha coccinea* L.). No symptom was noticed on raspberry (cv. Willamette) and blackberry (cv. Thornfree) shoots artificially inoculated.

The reisolation of the pathogen using necrotic tissues taken from the shoots infected were successful. Slide agglutination test carried out with reisolates obtained was positive.

Bacteriological characteristics of the strains investigated

On sucrose nutrient agar (SNA) the colonies were round, cream, large, convex and mucoid indicated levan formation. After 2–3 days of the development on Crosse-Goodman (CG) selective medium the colonies were pearly-white, high-domed, crateorous and mucoid. All strains investigated developed in a liquid medium with 5% NaCl, but not in a medium with 7% NaCl *(Table 1).*

The strains were oxidase negative and catalase positive; starch and tween 80 hydrolysis as well as the methyl-red test and hydrogen sulphide production from cysteine were negative; VP test (acetoin production) was positive *(Table 1).*

Lactose metabolism was negative and glucose, fructose, sucrose, xylose, mannitol, sorbitol, inositol and glycerol were positive *(Table 1).* Alkali production from acetate, citrate, D (–) tartrate and cis-aconitic acid was positive *(Table 1).*

Discussion

First appearence of the *E. amylovora* pathogen in Yugoslavia was observed in the middle of 1989 (Arsenijević et al., 1991; Panić and Arsenijević, 1993, 1996). Many diseased pear and quince trees were eradicated because fire blight spread quickly and appeared in numerous orchards and localities, with the tendency of further expansion. In 1993 the high degree of infection was also noticed on mespil in many districts. Up to 1995 the disease on apple trees was sporadic without strong infection. However, during 1995, 1996 and 1997 the fire blight symptoms outbraked suddenly and spread in numerous orchards and localities where various apple cultivars were growing (Arsenijević et al., 1996).

Too late and strong infection on many apple cultivars six years after the first disease appearance (1989) on pear and quince susceptible cultivars, observed in Yugoslavia was difficult to explain. Therefore, it was the main reason for parallel investigation of the *E. amylovora* strains originating from apple, pear and quince trees isolated in Yugoslavia, England and Canada *(Table 1).* As it was shown neither cultural nor biochemical differences were noticed among the strains investigated *(Table 1).* With regards to the pathogenicity all isolates induced HR on herbaceous plants. In immature fruits tissue test all pome isolates caused necrotic lesions with exudate oozing. No differences were also noticed in degree of the pathogenicity to apple, pear, quince and firethorn in shoot inoculation test. On raspberry and blackberry shoots artificially inoculated, no fire blight symptoms caused by any isolate studied was observed.

In the USA a group of specific *E. amylovoro* strains were isolated from raspberry and blackberry tissues and they were classified as different *Rubus* strains (Starr et al., 1951; Ries and Otterbacher, 1977). Norelli et al. (1984) reported that the *E. amylovora* strains from the *Rubus* species are host specific parasiting only *Rubus* plants but not

Malaceae. Recent investigations carried out in Belgium (Garbeva et al., 1996) pointed out also that *E. amylovora* strains originated from the *Rubus* plants were unable to cause fire blight symptoms on malaceous seedlings and shoots.

Judging on the results obtained it can be concluded that all the investigated strains isolated in Yugoslavia, Canada and England from pome fruits (apple, pear and quince) showed homogenous characteristics in all test performed *(Table 1).* The homogeneity among *Erwinia amylovora* strains originating from pomaceous and ornamental plants was registered by Vantomme et al. (1982), Shoeib et al. (1987), Brown et al. (1996), Kim et al. (1996).

Therefore, the *E. amylovora* isolates responsible for the strong infection of the apple trees in Yugoslavia does not consist a special strain adapted to Yugoslavian conditions and the apple cultivars growing in this area.

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Occurrence and Spread of Fire Blight Pathogen *(Erwinia amylovora)* on Spontaneous and Ornamental Plants in Yugoslavia

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During a ten-year-investigation on the occurrence and spread of fire blight *(Erwinia amylovora)* pathogen on spontaneous and ornamental plants in Yugoslavia, the pathogen was found only on hawthorn, wild pear and firethorn. No bacterium was isolated from others species among these two groups of hosts.

Keywords: Fire blight, *Erwinia amyvolora,* ornamental plants, spontaneous flora.

By its frequent occurrence and infection of high intensity *Erwinia amylovora* (Burrill) Winslow et al. drew attention in Yugoslavia mostly as a pathogen of Pomaceous fruit trees. At first it was found on quince and pear and later on medlar and apple trees (Arsenijević et al., 1991; Panić and Arsenijević, 1993, 1996).

Frequent and severe occurrence of *Erwinia amylovora* on Pomaceous fruit trees in Yugoslavia, and weak or none on ornamentals and on plants from spontaneous flora (except hawthorn) drew our attention to try to find pathogen presence also on other ornamentals and spontaneously growing species and to prove its occurrence experimentally.

Materials and Methods

The plant species examined and samples collecting

Between 1989 and 1999 various plant species were examined and samples for bacteriological tests in laboratory were collected. All samples from spontaneous and ornamental flora resembling to fire blight symptoms were collected.

Isolation of the pathogen

The isolation of the bacterium was carried out by usual procedure on sucrose nutrient agar (SNA) medium (Klement et al., 1990). No bacterial colonies growth after the isolations of the pathogen proved the lack of *E. amylovora* in the tissues of the plant species investigated, although the symptoms similar to fire blight bacterium were present among certain species of the samples collected. Additional isolation of the fungi on potato dextrose agar (PDA) was carried out in order to obtain reliable data.

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Check of pathogenicity and identification of the bacterium

Isolates of *E. amylovora* were tested by using basic methods of identification such as: tobacco (Klement, 1963), Pelargonium hypersensitivity (Arsenijević et al., 1991), Gram differentiation using 3% KOH (Suslow et al., 1982; loc. cit. Arsenijević, 1997), growth characteristics on Crosse-Goodman's (CG) medium, lack of fluorescence on King's medium B, inoculation of immature pear (cv. Williams) and plum (cv. Stanley) fruits and the hosts from which the bacterial strains were isolated (Klement et al., 1990; Arsenijević, 1997). Slide agglutination test was carried out by the standard procedure (Klement et al., 1990) using immune serum produced by authentic *E. amylovora* strain.

Results

Spontaneous flora (Fig. 1)

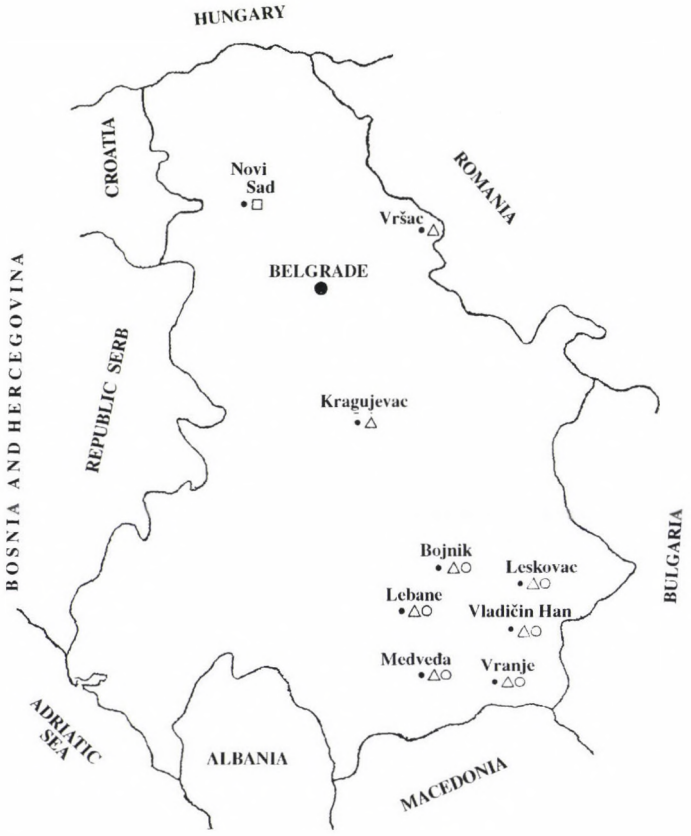


Fig. 1. *E. amylovora* on hawthorn (△), firethorn (□) and wild pear (○) in Yugoslavia

Among the samples collected from spontaneous flora only wild pear (*Pyrus piraster* Burgs.) and hawthorn (*Crataegus monogyna* Jacq.) manifested positive results. From the samples of other species: mountainash – *Sorbus domestica* L., sloe plum – *Prunus spinosa* L., wild apple – *Malus silvestris* Miller, etc. there was no growth of bacterial colonies or the colonies of fungi were grown on PDA.

In the main tests of identification the isolates of bacteria originating from wild pear and hawthorn manifested all properties typical for *E. amylovora.* The colonies on SNA medium were whitish-cream, levan type and crateorous on CG medium; the bacteria were Gram negative, asporogeneous and rod-shaped. In contrast to the *Pseudomonas syringae* pv. syringae (J-50) check strain, isolates of *E. amylovora* did not produce green fluorescent pigment on King' s medium B, formed droplets of bacterial exudate on immature pear and plum fruitlets artificially inoculated and they reacted positively with *E. amylovora* antiserum in agglutination test.

Ornamental plants (Fig. 1)

Many species of ornamental plants were investigated, whether they were cited in the literature as *E. amylovora* hosts or not (june-berry – *Amelanchier ovalis* Medic., flowering japanese quince – *Chaenomeles japonica* (Thumb.) Lindr. ex Spach, rock spray cotoneaster – *Cotoneaster horisontalis* Decne., loquat – *Eryobotrya japonica* (Thumb.) Lindl., box-free – *Buxus sempervirens* L., golden bell – *Forsythia intermedia* Zab., cherry laurel – *Prunus laurocerasus* L., firethorn – *Pyracantha coccinea* Roem.), because, they express fire blight like symptoms. However, *E. amylovora* was isolated only from firethorn (*Pyracantha coccinea* Roem.).

The pathogen was found for the first time on firethorn in 1997 in the center of Novi Sad city, where these plants were cultivated for ornamental purpose. In the following year (1998) the fire blight disease was registered in other part of the city in another two localities. All bacterial isolates originating from firethorn manifested main characteristics typical for *E. amylovora* bacterium as it was given in the chapter "Spontaneous flora".

However, in the absence of *E. amylovora* on SNA medium, several fungal colonies appeared on PDA: *Leucostoma, Nectria, Monilinia, Phomopsis,* etc. were isolated from necrotic tissues of the species, showing fire blight like symptoms.

Discussion

Fire blight *(E. amylovora)* bacterium is widely spread and economically important pathogen of Pomaceous trees in Yugoslavia (Arsenijević et al., 1991; Panić and Arsenijević, 1993, 1996). Among spontaneous flora, the infection was proved for the first time on hawthorn and wild pear in 1991 (Panić and Arsenijević, 1996).

On ornamental plants cultivated in Yugoslavia, *E. amylovora* was not found. Only in the summer of 1997, weak infection was found on firethorn in the center of Novi Sad (Gavrilović and Arsenijević, 1998). The infection spread slowly, and the expansion of the pathogen was stopped by mechanical measures. However, fire blight on hawthorn

and wild pear occurred more frequently *(Fig. 1)* in different localities (Arsenijević and Panić, 1996; Arsenijević and Gavrilović, 1998).

It can be concluded that hawthorn, in addition to Pomaceous plants (pear, quince, apple and medlar trees), is a frequent and susceptible host of *E. amylovora* in Yugoslavia. This is also proved in other countries (Graberg, 1993; Jorgensen, 1978; Billing 1981, etc.) On the contrary, except for Novi Sad, firethorn as well as many other ornamental plants were free from the fire blight disease (Arsenijević and Panić, 1996).

However, *Cotoneaster, Crataegus, Pyracantha, Stranvaesia* and *Sorbus* in many countries manifested extreme susceptibility to *E. amylovora.* Due to this, many ornamental shrubs in parks and gardens or elsewhere have to be eradicated and destroyed (Garrett, 1990; Meijneke, 1975; van der Zwet and Keil, 1979; IWG Reporter, 1991)

Such a rare and weak occurrence of *Erwinia amylovora* pathogen on firethorn and frequent and severe infection on Pomaceous trees and hawthorn in Yugoslavia deserves full attention. Therefore, further investigations of *E. amylovora* on ornamentals and spontaneous flora, are of great interest.

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Development and Genetic Analysis of Common Wheat Introgressive Lines Resistant to Leaf Rust

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Transfer of genetic material from tetraploid species, *Triticum timopheevii* Zhuk, resistant to diseases into common wheat *Triticum aestivum* L. has been carried out. Six common wheat cultivars were used. The process of hybrid forms stabilization have been studied in details; identification of *Triticum timopheevii* genetic material in genome of introgressive lines ($2n = 42$) resistant to leaf rust (*Puccinia triticina* Erikss.) has been carried out. Donors of rust resistance have been developed and the genetic control of the character has been studied. The resistance of obtained lines has been shown to be due to *Lr* genes, non-allelic to effective *Lr* genes from world collection.

Keywords: *Triticum timopheevii* Zhuk, introgressive lines, common wheat, resistance to leaf rust.

Development of cultivars resistant to diseases – one of the principal directions in up-to-date breeding. Wheat, being one of the main agricultural crops, is subjected to a considerable yield lost (30–50%) after diseases attack in epiphytotic years. Every year lost only from rust consists 5–10% (Mikhailova and Gultjaeva, 1998).

The most reliable and ecologically safe method of disease control is breeding and growing of resistant cultivars. Among common wheat relatives, in the world centers of its origin, several species are available, carrying the genes of resistance to diseases. Literature data indicate of many attempts to transfer such genes into commercial cultivars (Dvorak, 1977; Knott and Dvorak, 1981; Sears, 1981; Gill and Raupp, 1987, etc.). Use of chromosome manipulation allowed to enrich essentially the wheat germplasm with resistant genes. But the resistant forms on the genetic base of different cultivars have been derived sufficiently accident.

The main aim of our investigation was to use the unique pool of resistant genes in tetraploid endemic species, *Triticum timopheevii* Zhuk and making it available for resistance breeding. For this purpose, it was necessary to develop the collection of stable introgressive lines of common wheat with chromosome number $2n = 42$, with expression of *Triticum timopheevii* Zhuk resistance genes and with considerable number of characters similar to common wheat. These donor lines may serve as a constant source of "needed" genes in breeding process.

Materials and Methods

The direct transfer of *Triticum timopheevii* Zhuk (genome formula AAGG, $2n = 28$) genes through hybridization is hampered because of poor crossability with common

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wheat *Triticum aestivum* L. (genome formula AABBDD, $2n = 42$), sterility of F_1 hybrids and instability of hybrids in early generations.. Only single backcrossing of F_1 hybrids with initial cultivar was used followed by self-pollinating. As recipients of *Triticum timopheevii* Zhuk *(T.t)* genetic material, six commercial common wheat cultivars were used: Saratovskaya 29 (S29), Novosibirskaya 67 (N67), Pyrotrix 28 (P28), Skala (Sk), Irtyshanka (Irt) and Tselinnaya 20 (Ts20). Hybrid plants in population derived from crossing of these cultivars as a female parent with *Triticum timopheevii* Zhuk were studied cytogenetically in F_1B_1–F_7B_1 generations. Cytologically stable plants were picked out, resistant to a field population of leaf rust which is rather constant in Siberian conditions (Mikhailova and Tyryshkin, 1994). Mains-Jackson scale of immunity was used for evaluation of resistanse.

For identification of *Triticum timopheevii* Zhuk genetic material transferred into common wheat the following methods have been used: C-banding technique, method of biochemical markers (Kalinina et al., 1987) and molecular markers (Leonova et al., 1999). The every year strict evaluation of resistance to rust was carried out for introgressive lines and their hybrids with initial and tester lines.

Results and Discussion

Earlier we discussed the possibility to obtain wheat lines resistant to diseases after crossing of three cultivars with *Triticum timopheevii* Zhuk (Budashkina, 1988). Further comparative investigations of hybrid populations (F_1B_1–F_7B_1) derived from crossing of six cultivars with this species, have shown the considerable influence of recipient genotype on intensity of genetic material transfer (Shkutina et al., 1998).

Using of biochemical markers have allowed to identify 9 from 14 chromosomes of *Triticum timopheevii* Zhuk haploid set (Kalinina et al., 1990). The following tendency have been established: the more chromosomes from two crossing species are engaged in bivalent pairing in F_1 hybrids, the more diversity of marked chromosome arms of donor species is presented in F_4B_1 offspring. Based on the data obtained the conclusion have been done that in the whole, the introgression of genetic material of *Triticum timopheevii* Zhuk goes effectively enough into this crossing combination. By C-banding of chromosomes in cytologically stable introgressive lines the transfer of alien genetic material has been shown to take place presumably according to the homology of entire chromosomes or their arms; translocations have been registered much more rarely (Badaeva et al., 1991). Chromosomes of the 7th homological group mostly were involved into rearrangements, of the 3rd – rather rarely.

Selection for resistance to rust have been made among the cytologically stable ($2n = 42$) lines picked out hybrid populations descended from different cross combinations. Data from *Table 1* demonstrated resistance to several races of both leaf and stem rust (Mikhailova, 1992, personal communication).

C-banding of resistance to leaf rust forms has shown that the greatest transfer was registered in 2nd and 6th homological group.

Table 1

Rust resistance in introgressive wheat lines

Variety, Line	Leaf rust							Stem rust							
	Populations			Races				Races						Populations	
	1*	2*	3*	15	25	77	192	11	17	21	34	40	117	2*	3*
S 29	4	3	3	–	4	4	4	4	4	4	4	4	3	4	4
753–1	0	0	0	0	0	0	0	0–2	0	0	4	x	0	3	3
778–3	0	0	0	0,1	0	0,1	0	–	4	0,2	0	4	3,4	0	3
797–1	0,1	0	0	0,1	0	0	0	4	0	0	4	3,4	4	3	3
829–1	0	1–2	1–2	0	0	0	0	x	0,1	0	0	–	–	0	3
842–2	0,1	0	0	0,1	0	0	0	x	3,4	0	0	4	0,2	1	2
T. tim	0	0	0	0	0,1	0	0	0,2	0–2	0	0,1	0	0,1	0,1	1

Notes: 1* – Novosibirsk; 2* – Derbent; 3* – Rostov
0, 1, 2 – resistance; 3, 4 – susceptibility

At present, there are about 80 introgressive lines in our collection, resistant to field population of leaf rust. Genetic analysis of their resistance is under study now *(Table 2)*. This character has been shown to be under control of 1–2 genes with different

Table 2

Genetical analysis of leaf rust resistance in introgressive wheat lines

Line derivation	Number of line studied	Segregation pattern (res : susc) in the F_2 hybrid populations (line x initial variety)							
		3 : 1	9 : 7	13 : 3	15 : 1	1 : 3	7 : 9	3 : 13	1 : 15
S 29 x T.t	28	–	8	–	–	10	10	2	1
N 67 x T.t	12	1	2	–	–	8	3	2	2
P 28 x T.t	15	–	–	–	–	4	–	5	6
Irt x T.t	14	9	3	–	3	–	1	–	–
Skala x T.t	5	2	1	2	1	1	1	–	–
Ts 20 x T.t	7	2	1	3	–	–	1	–	–
Σ	81	14	15	5	4	23	16	9	9

interactions between them. In some lines, the character of genetic control changes in dependence of season. Most of lines show the stability of resistance. Genetic analysis of sublines derived from some lines confirms this statement *(Table 3)*.

Tests on allelism have shown that *Triticum timopheevii* Zhuk genes introgressed into common wheat providing it with resistance to the leaf rust are not allelic to known effective genes of world collection, such as *Lr9, Lr19, Lr23, Lr24 (Table 4)*.

Thus, the developed collection of introgressive lines may serve as a source of new genes for resistance to rust in common wheat breeding. Nowadays, the approaches are elaborating to map these genes on definite chromosomes.

Table 3

Segregation in hybrid populations F_2 (subline x initial cultivar)

Cross combination	Phenotypes ratio (res :susc)		Segregation type	$\chi 2$	P
	actual	theoretical			
157-2 x Sk	36 : 13	37 : 12	3 : 1	0.11	0.75–0.5
157-3 x Sk	27 : 7	21 : 7	3 : 1	0	
157-4 x Sk	34 : 8	32 : 10	3 : 1	0.53	0.5–0.25
157-5 x Sk	49 : 13	47 : 15	3 : 1	0.36	0.75–0.5
140-1 x Irt	19 : 48	17 : 50	1 : 3	0.32	0.75–0.5
140-2 x Irt	16 : 64	20 : 60	1 : 3	1.07	0.5–0.25
140-3 x Irt	26 : 39	28 : 37	1 : 3	0.25	0.75–0.5

Table 4

Segregation in the F_2 hybrid populations (line x tester) for resistance (resistant : susceptible)

Line	Testers			
	Lr 9	Lr 19	Lr 23	Lr 24
811	82 : 7	36 : 15	19 : 45	87 : 3
78	59 : 13	42 : 33	52 : 36	62 : 25
747	69 : 6	67 : 11	62 : 11	67 : 10
673	24 : 10	61 : 12	25 : 24	63 : 13
692	66 : 9; 77 : 8	80 : 4	21 : 5	77 : 9
699	86 : 11	74 : 12	61 : 29	70 : 8; 77 : 8
640	78 : 3	61 : 7; 47 : 10; 66 : 11	39 : 26	67 : 4; 60 : 15
64	61 : 7	44 : 7	49 : 10	52 : 30
82	56 : 11	52 : 7	50 : 7	53 : 0
191	50 : 9	51 : 3	49 : 4	65 : 3
141	67 : 2	63 : 3	35 : 20	19 : 21

The size and position of introgressed *Triticum timopheevii* Zhuk fragments have been evaluated using microsatellite markers (SRR) (Leonova et al., 1999) in three resistant lines derived on the bases of S29 cultivar. SRR markers with known chromosome localization in common wheat were used (Röder et al., 1998). It was shown that in all three lines the large parts of 2A and 2B chromosomes are involved in substitution.

The resistance in these lines may be due to *Triticum timopheevii* Zhuk genes introgressed in 2A and 2B chromosomes. This is in agreement with literature data in the localization of considerable number of genes in chromosomes of 2nd homological group (McIntosh et al., 1995).

Further analysis of developed introgressive lines will allow to enlarge our knowledge about genetics of resistance and to enrich the genetic map of common wheat.

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Differential Induction of Chitinase and β-1,3-Glucanase in Rice in Response to Inoculation with a Pathogen *(Rhizoctonia solani)* and a Non-Pathogen *(Pestalotia palmarum)*

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Rice leaf sheaths were inoculated with *R. solani* (pathogen) and *P. palmarum* (non-pathogen) and were analyzed for the accumulation of pathogenesis-related (PR) proteins. Inoculation of rice plants with *R. solani* and *P. palmarum* resulted in a marked increase in activities of chitinase and β-1,3-glucanase. The levels of both enzymes were higher in incompatible interactions than in compatible interactions. Western blot analysis indicated that two proteins with molecular weights of 33 and 35 kDa cross-reacting with barley chitinase antibody were induced in rice in response to inoculation with *R. solani*. The appearance of these chitinases was correlated with increase in enzyme activity.

Keywords: Compatible interaction, incompatible interaction, *Pestalotia palmarum, Oryza sativa,* PR-proteins, *Rhizoctonia solani.*

Plants, in general, possess several structural and biochemical defense mechanisms to overcome the infection by pathogens. Some of the chemical compounds are antifungal and are constitutive, existing in healthy plants. For example phenolics, phenolic glycosides, unsaturated lactones, saponins, cyanogenic glycosides, glucosinolates, 5-alkylated resorcinols and dienes (Osbourn, 1996). In addition to the preformed antimicrobial compounds, several defense mechanisms are induced in response to infection by potentially pathogenic microorganisms. These inducible defense responses include hypersensitive cell death (Levine et al., 1994), production of phytoalexins (Kuc and Rush, 1985), accumulation of Pathogenesis-Related (PR) proteins (Velazhahan et al., 1998, 2000), induction of oxidative burst (Baker and Orlandi, 1995), lignification (Ride, 1975) and cross-linking of wall glycoprotein (Bradley et al., 1992). The speed of expression of these defense genes determines the susceptible or resistant state of the host plant.

In recent years, much research has been focussed on PR-proteins (Datta and Muthukrishnan, 1999). The PR-proteins are a group of extracellular proteins that are induced by different phytopathogens such as fungi, bacteria and viruses, fungal elicitors and certain chemical inducers such as salicylic acid, polyacrylic acid and 2,6-dichloroisonicotinic acid (Bol et al., 1990; Uknes et al., 1992). These proteins have been extensively studied in tobacco and are identified in many monocotyledonous and dicotyledonous plants (Bol et al., 1990; Linthorst, 1991). Several studies suggested that the expression of PR-proteins correlated with the development of systemic acquired resistance

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(SAR) in plants (Ward et al., 1991; Uknes et al., 1992). Marked differences in the timing of activation of chitinase and glucanase genes in plants depending on compatibility or incompatibility of the interaction have been reported (Ward et al., 1991; Daugrois et al., 1990). We studied the changes in PR-proteins in rice in response to infection by *Rhizoctonia solani,* the rice sheath blight pathogen (a compatible interaction) and *Pestalotia palmarum* (nonpathogenic to rice: an incompatible interaction).

Materials and Methods

Plant material

The rice cultivar IR50 was used in all experiments. The seeds were obtained from Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore. The plants were grown in 25 cm diameter earthern pots containing rice field soil in a greenhouse according to standard methods.

Fungal culture and inoculation

Rhizoctonia solani was isolated from sheath blight infected rice plants and maintained on Potato Dextrose Agar (PDA) medium. The fungus, *Pestalotia palmarum* was isolated from grey leaf spot infected coconut leaves and maintained on PDA medium. Individual tillers of 35 to 40 day old plants were inoculated with *R. solani* and *P. palmarum* by placing an agar block containing the mycelium on the leaf sheath and wrapping them with parafilm to maintain high humidity. At various times after inoculation, plant samples were collected and frozen in liquid nitrogen before protein extraction.

Assay of chitinase

Rice leaf sheaths (1 g) were homogenized in 5 ml of 0.1 M sodium citrate buffer (pH 5.0) using a chilled pestle and mortar. The homogenate was then centrifuged for 10 min at 10,000 g at 4 °C and the supernatant was used in the enzyme assay. Colloidal chitin was prepared from crab shell chitin (Sigma) according to Berger and Reynolds (1958). The commercial lyophilized snail gut enzyme (Helicase, obtained from Sepracor, France) was desalted as described by Boller and Mauch (1988). For the colorimetric assay of chitinase 10 µl of 1 M sodium acetate buffer (pH 4.0), 0.4 ml of enzyme extract and 0.1 ml colloidal chitin (1 mg) were pipetted into a 1.5 ml Eppendorf tube. After 2 h at 37 °C, the reaction was stopped by centrifugation at 1,000 g for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 µl of 1 M potassium phosphate buffer (pH 7.1) and incubated with 20 µl desalted snail gut enzyme for 1 h. The resulting monomeric N-acetylglucosamine (GlcNAc) was determined according to Reissig et al. (1955) using internal standards of GlcNAc in the assay mixtures for calculations. Enzyme activity was expressed as nmol GlcNAc equivalents min^{-1} g^{-1} fresh weight.

Assay of β-1,3-glucanase

β-1,3-glucanase activity was assayed by the laminarin-dinitrosalicylate method (Pan et al., 1991). Rice leaf sheaths (1 g) were extracted with 5 ml of 0.05 M sodium acetate buffer (pH 5.0) by grinding at 4 °C using a pestle and mortar. The extract was then centrifuged at 10,000 g for 15 min at 4 °C and the supernatant was used in the enzyme assay. The reaction mixture consisted of 62.5 µl of 4% laminarin and 62.5 µl of enzyme extract. The reaction was carried out at 40 °C for 10 min. The reaction was then stopped by adding 375 µl of dinitrosalicylic reagent and heating for 5 min on a boiling water bath. The resulting coloured solution was diluted with 4.5 ml of distilled water, vortexed and its absorbance at 500 nm was determined. Enzyme activity was expressed as nmol min^{-1} g^{-1} fresh weight.

Western blot analysis

Proteins were extracted from control and *R. solani*-inoculated leaf sheaths by grinding 1 g of tissues with 5 ml of 0.1 M phosphate buffer, pH 6.5 containing 0.5 mM phenylmethylsulphonyl fluoride as described by Velazhahan et al. (1998). Proteins (100 µg) in aliquots of extracts were separated on 12% SDS-PAGE (Laemmli, 1970) and electroblotted onto polyvinylene difluoride (PVDF) membrane (pore size 0.45 µm, Bio-Rad) using BioRad semidry transfer cell in accordance with the manufacturer's instructions. Western blotting was carried out as described by Winston et al. (1987) with an antiserum raised against barley chitinase. The barley chitinase antiserum was a gift from Dr. S. Muthukrishnan, Kansas State University, USA. Apparent molecular mass of proteins was determined by comparison with molecular weight standards (Rainbow markers, Amersham Pharmacia, USA). Protein concentrations were determined by Bradford assay (Bradford, 1976).

Results

A significant increase in chitinase activity was observed in rice leaf sheaths after inoculation with *P. palmarum*. The maximum enzyme activity was recorded 2 days after inoculation subsequently the enzyme activity declined but remained significantly at higher levels when compared with control *(Fig. 1)*. A 2-fold increase in chitinase activity occurred 2 days after inoculation with *P. palmarum*. *R. solani*-inoculated rice leaf sheaths showed sudden increase in chitinase activity 1 day after inoculation, subsequently the enzyme activity declined but the enzyme levels in infected leaf sheaths were significantly higher at all sampling stages when compared with control. To know whether the increase in chitinase activity in *R. solani*-inoculated rice leaf sheaths is due to *de novo* synthesis or due to post-translational modification of the pre-existing inactive enzyme, the crude proteins from *R. solani*-inoculated rice leaf sheaths at different time intervals were analyzed by Western blot analysis using barley chitinase antibody. A 35-kDa chitinase appeared 12 h after inoculation *(Fig. 2)*. The induction of the chitinase was prominent 24–48 h after inoculation. Another chitinase with a molecular weight of 33 kDa was detected 24–48h after inoculation.

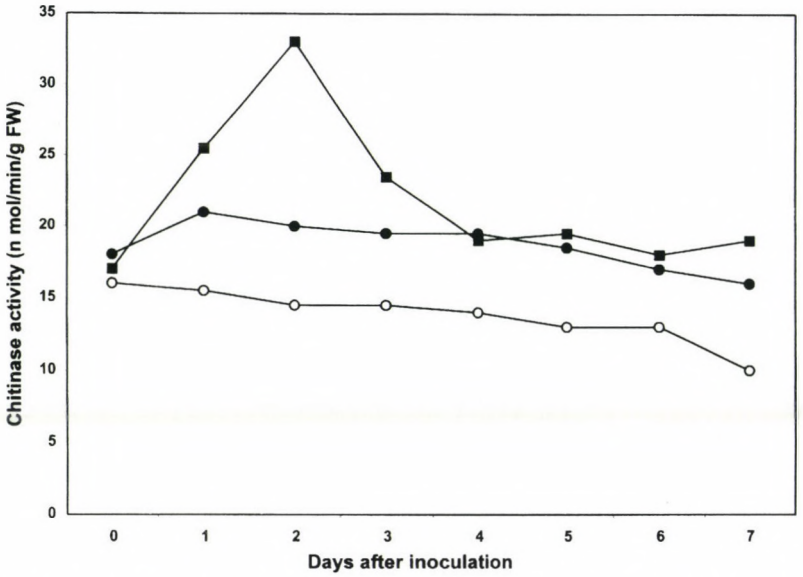


Fig. 1. Changes in chitinase activity in rice in response to inoculation with *Rhizoctonia solani* and *Pestalotia palmarum.* Chitinase activity in protein extracts of control (– O –), *R. solani*-inoculated (– ● –) and *P. palmarum*-inoculated (– ■ –) rice leaf sheaths was measured colorimetrically. Least significant difference to compare any two means at P = 0.05 is 3.39

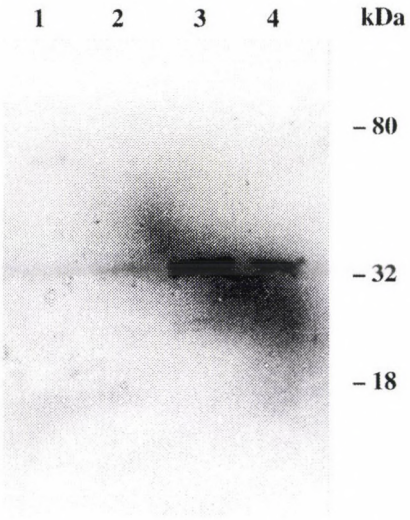


Fig. 2. Induction of chitinase in rice in response to inoculation with *Rhizoctonia solani.* Aliquots (100 µg) of proteins extracted from control (lane 1) and *R. solani*-inoculated rice leaf sheath (lanes 2–4) were analyzed by Western blotting after SDS-PAGE, using a barley chitinase antiserum. Lane 2; 12 h after inoculation; Lane 3; 24 h after inoculation; Lane 4; 48 h after inoculation. Sizes of marker proteins are indicated on the right

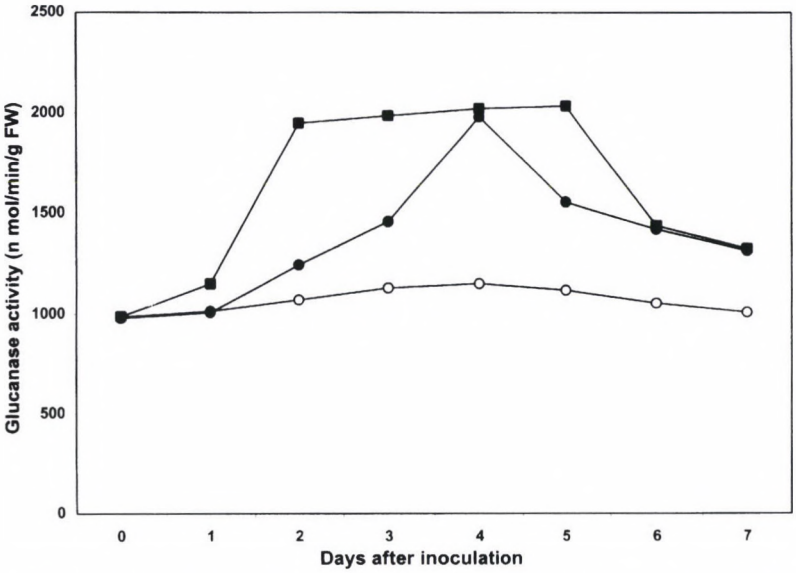


Fig. 3. Changes in β-1,3-glucanase activity in rice in response to inoculation with *Rhizoctonia solani* and *Pestalotia palmarum.*
Glucanase activity in protein extracts of control (– O –), *R. solani*-inoculated (– ●–) and *P. palmarum*-inoculated (– ■ –) rice leaf sheaths was measured colorimetrically. Least significant difference to compare any two means at P = 0.05 is 10.45

β-1,3-glucanase activity steadily increased in *R. solani* inoculated rice leaf sheaths from 2 days onwards and reached the maximum 4 days after inoculation *(Fig. 3).* The enzyme activity was significantly at higher levels at all sampling stages when compared with control. Rice leaf sheath responded to inoculation with *P. palmarum* with a rapid increase in glucanase activity from 1 day onwards and reached the maximum level at 5 days after inoculation. The levels of glucanase activity were higher in incompatible than in compatible interactions.

Discussion

Chitinases and β-1, 3-glucanases have been suggested to be involved in resistance of plants against fungal pathogens (Goy et al., 1992; Pan et al., 1991). Chitinases and β-1,3-glucanases have the potential to hydrolyze chitin and β-1,3-glucan respectively, which are major components of fungal cell walls, leading to direct inhibition of growth of several fungi (Schlumbaum et al., 1986; Leah et al., 1991). It has been demonstrated that both chitinases and β-1,3-glucanases from pea tissues acted synergistically in partial degradation of isolated fungal cell walls (Mauch et al., 1988). In addition chitinases and

glucanases play an indirect role in stimulating plant defense by releasing oligosaccharides from the fungal cell walls by their enzymatic action which act as "elicitors" or inducers of several defense genes (Ryan, 1987; Keen and Yoshikawa, 1983). High constitutive levels of β-1,3-glucanase activity was found to be associated with leaf rust resistance in wheat (Anguelova et al., 1999). Similarly, higher levels of β-1,3-glucanase activity were recorded in resistant muskmelon plants than in susceptible plants (Ward et al., 1991).

Early increase in chitinase and glucanase activities has been correlated with resistance of plants against pathogens. In the resistant *Brassica napus* cultivar, the level of chitinase mRNA was three-folds higher than in the susceptible cultivar one day after inoculation (Rasmussen et al., 1992). In the present study, the time-courses of chitinase and glucanase activities in response to infection by *R. solani* and *P. palmarum* were examined. Inoculation of rice leaf sheaths with *R. solani* and *P. palmarum* resulted in a marked increase in activities of both chitinase and glucanase. The accumulation of both chitinase and glucanase was more in rice tissues infected with *P. palmarum.* The induction of glucanase in rice was much faster in incompatible interaction. Esnault et al. (1993) observed high-level accumulation of a 0.8 kb transcript encoding a PR-protein 6 to 30 h post infection in the incompatible interaction of alfalfa with *Pseudomonas syringae* pv. *pisi.* In contrast, the 0.8 kb transcript was undetectable in the compatible interaction with *Xanthomonas campestris* pv. *alfalfae,* even 30 h after infection. Similarly maximum accumulation of chalcone isomerase and isoflavone reductase transcripts was recorded 6 h post infection in incompatible interactions whereas in the compatible interaction, the induction of these transcripts was delayed until 25–30 h post infection. Similarly accumulation of chitinase mRNA was reported to start earlier in an incompatible interaction in bean – *Colletotrichum lindemuthianum* interaction (Bell et al., 1986; Hedrick et al., 1988). Daugrois et al. (1990) reported that the increase in β-1,3-glucanase and chitinase activities in bean were much faster in compatible interactions. In soybean roots infected by *Phytophthora megasperma* f. sp. *glycinea,* accumulation of PAL and chitinase mRNAs started earlier in incompatible than in compatible interaction (Habereder et al., 1989). However, Punja and Zhang (1993) reported higher levels of chitinase activity in infected susceptible plants than in resistant plants. They attributed this to stress levels induced by higher fungal biomass in susceptible plants. The results of the present study indicated that the accumulation of chitinase and β-1,3-glucanase was more pronounced in incompatible rice – *P. palmarum* interaction suggesting that these hydrolytic enzymes may play a role in resistance of rice to *P. palmarum.*

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Monitoring the Susceptibility of Two *Leptinotarsa decemlineata* (Say) Populations to *Bacillus thuringiensis* ssp. *tenebrionis*

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Standardized laboratory bioassays were carried out at the Institute for Biological Control of the Federal Biological Research Center for Agriculture and Forestry to determine the susceptibility of two Colorado potato beetle (CPB) populations to *Bacillus thuringiensis* ssp. *tenebrionis (B.t.t.).* The beetles were collected from fields near Darmstadt/Hessen, and Michelfeld/Baden-Württemberg. The Michelfeld population, collected from an ecological farm, was sprayed since 1993, once or twice a year with NOVODOR FC, a formulation of *B.t.t.* The Darmstadt population was never sprayed with *B.t.t.* before. Our studies showed that the susceptibility of the larvae (LD_{50} values) of the two populations did not differ each other significantly. Although resistance of CPB to *B.t.t.* under laboratory conditions has already been demonstrated, our presumption is that the resistance development of CPB on the field is probably much slower when the farmer is using crop rotation.

Keywords: *Bacillus thuringiensis* ssp. *tenebrionis,* Colorado potato beetle, population, resistance, susceptibility.

Bacillus thuringiensis ssp. *tenebrionis (B.t.t.)* was discovered in Darmstadt, Germany, in 1982 (Krieg et al., 1983, 1984). The δ-endotoxin of the novel *Bacillus thuringiensis* strain (pathotype C) is especially active against leaf beetles (Chrysomelidae), but ineffective against Lepidoptera, Diptera, or other insect orders. In the following years Herrnstadt et al. (1986) reported about another strain with coleopteran activity, *Bacillus thuringiesis* ssp. *san diego,* which was later found to be identical to *B.t.t.* (Krieg et al., 1987). The chrysomelid active character of *B.t.t.* has allowed scientists to start laboratory and field experiments with Colorado potato beetle (CPB).

The results of the field experiments in Germany showed that *B.t.t.* could play an important role in the future as a successful tool for the biological control of larvae of the CPB (e.g. Langenbruch et al., 1985; Langenbruch and Riethmüller, 1990; Langenbruch and Hommel, 1991; Langenbruch, 1992; Gürlich and Langenbruch, 1994). The scientific results of American researchers were similar. Ferro and Gelernter (1989), Zehnder and Gelernter (1989), Ferro and Lyon (1991) and Hough-Goldstein et al. (1991) reported about successful biological control with different formulations of *B.t.t.*

The δ-endotoxin of *B.t.t.* is an environmentally sound alternative to broad spectrum synthetic insecticides to control CPB. Recently, products based on *B.t.t.* and licenced as a biological insecticide are available on the markets of most countries in Europe and

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North-America (NOVODOR FC). Biological insecticides are mainly used IPM growers, ecological farmers and ecological thinking hobby-gardeners. The Cry3A δ-endotoxin has already been genetically engineered into potatoes, too (Gasser and Fraley, 1989). These modificated potatoes producing δ-endotoxin against the CPB are already available on the American market.

Whalon et al. (1993) first reported about resistance of CPB against *B.t.t.* under laboratory conditions. After 12 generations of *B.t.t.* selection, the selected CPB strain was 59 times more resistant than the unselected CPB strain, and 24–35 times more resistant than other susceptible, or insecticide-resistant strains. Rahardja and Whalon (1995) investigated the genetic inheritance of CPB resistance to *B.t.t.* δ-endotoxin. They suggested that the resistance might be caused by more than one locus. The resistance was not stabile. When the selection pressure was removed, the resistance level of the selected colony decreased after five generations. Trisyono and Whalon (1997) reported about the fitness costs of resistance to *B.t.t.* In their experiments the resistant females produced 60% fewer eggs than susceptible ones.

Is the development of *B.t.t.* resistance to be expected in the field (e.g. on isolated farms using NOVODOR for a long time)? Must ecological farmers be afraid to loose an effective biological tool against CPB soon? Can the susceptibility of the CPB larvae towards *B.t.t.* be measured with standardised methods?

Materials and Methods

Colony and bioassay

At the end of May, 1999, CPBs were collected from two German farms. One of the farms (Michelfeld / Baden-Württemberg) produces agricultural products ecologically. Therefore, NOVODOR FC has been used once or twice a year, for a period over 5 years, to control CPB. The other farm, where imagines were collected, is found 10 km from Darmstadt / Hessen. *B.t.t.* was never sprayed here before. The two colonies, which were maintained in the greenhouse of the institute, allowed to carry out bioassays to determine the susceptibility of the L_1 larvae towards *B.t.t.*

In laboratory bioassays, according to Riethmüller and Langenbruch (1989), L_1 larvae 24 hrs after hatching and holding on potato leaves tested to determine the LD_{50}. 10 small leaf discs (d = 3 mm), each bioassay contained five treatments, in three repetitions treated with 2 microliter distilled water, and with four ascending contrentrations of the product NOVODOR FC, which contains according to the German index of plant protection products 20 g *B.t.t.*/kg. Every assay was repeated three times. After 48 hours on the treated leaf discs, the larvae received untreated potato leaves until the end of the experiment. After six days the dose-mortality-effect was determined. The temperature during the experiment was 24±1 °C, the photoperiod 16:8 LD.

Data analysis

Data were analysed by probit regression (Finney, 1971); Abbott's (1925) formula was used to correct the mortality. Bioassays with more than 10% control mortality were not evaluated. With probit-regression the LD_{50} value with the 95% fiducial limits, and the slope of the regression line with the standard deviation, could be computed. Eight bioassays were carried out with the Darmstadt population, and seven bioassays with the Michelfeld population. On the one hand, the LD_{50} values of the populations could be compared with *t*-test, but this method does not take the 95% fiducial limits into consideration. On the other hand, there is a possibility by the probit-regression to compare two regression lines after computing a new common slope. With this method to determine an estimate of relative medium potency (and 95% fiducial limits) the two populations are comparable (Daum and Killcreas, 1966).

Because of the asynchronous egg laying of the imagines in the different CPB strains it was not possible to carry out the bioassays parallel. Therefore, summarising the replications of the populations ($8 \times 3 = 24$ replications by the Darmstadt population, and $7 \times 3 = 21$ replications by the Michelfeld population) seems to be a right way. This method does not take the different larval body weight into consideration, but because of the large number of investigated larvae (about 1000/population) it is a sure method. The larval body weight could have influenced the LD_{50}. Therefore, the larval body weight of both populations should previously be compared. Because all other circumstances of the bioassays were similar, summarizing of the replications is possible when no significant differences between the two groups are found.

Results and Discussion

Table 1 shows the larval body weight and the LD_{50} values computed with the probit regression analysis, the 95% fiducial limits of LD_{50} values, and the slope with the standard error for every bioassay.

As *Table 2* shows using the t-test for statistical analysis, with respect to body weight, no significant difference was found between the populations ($t = 1.149$; $df = 13$; $P = 0.271$). The fact that the larval body weight did not differ from each other can be easily explained. The larvae used for bioassays were held 24 hours after hatching on untreated potato leaves. All of them had the possibility to consume enough. The fact that the larval body weights are homogeneous in the two groups, allowed us concentrate all replicates.

By comparing the LD_{50} values with t-test the differences were also not significant ($t = 0.059$; $df = 13$, $P = 0.954$). This test cannot take 95% fiducial limits into consideration, so, before rating this result, the other evaluation method (concentrating of all replicates) should be completed.

Table 3 contains the data of the probit regression after the concentration of all replicates. The average body weight of the Darmstadt population was 1.13 mg, the Michelfeld population 1.31 mg. The computed LD_{50} values hardly differred from each other: 2.38×10^{-6} mg *B.t.t.*/larva of the Darmstadt population, and 2.12×10^{-6} mg *B.t.t.* of larva by the Michelfeld population. An interesting effect is that the Michelfeld population with the heavier larval body weight is somewhat more susceptible to *B.t.t.* than the Darmstadt population.

Table 1

Body weight, LD_{50} value and spole for each bioassay of the tested populations

Number of bioassay	Body weight (mg/larva)	LD_{50} (mg *B.t.t.*/larva) x 10^{-6}	LD_{50} 95% fiducial limits	Slope ± SE
Darmstadt				
	0.85	2.50	1.93–3.17	3.48±0.68
	0.93	2.87	2.04–4.56	2.43±0.49
	0.95	2.14	1.47–2.97	2.13±0.48
	0.97	1.92	1.41–2.46	2.96±0.57
	1.00	2.67	2.22–3.25	3.89±0.60
	1.16	2.14	1.70–2.67	2.82±0.45
	1.25	2.65	1.83–4.01	3.11±0.70
	1.93	2.17	1.65–2.80	2.49±0.45
Michelfeld				
	1.36	1.74	1.25–2.28	2.13±0.40
	0.85	2.69	2.08–3.49	2.82±0.52
	1.21	1.77	1.40–2.21	2.98±0.49
	1.25	1.09	0.70–1.44	2.37±0.48
	1.40	2.11	1.56–2.82	2.11±0.40
	1.50	2.05	1.43–2.92	1.79±0.40
	1.61	5.42	0.37–12.7	1.73±0.44

Table 2

Mean of larval body weight and LD_{50} per population

Population	Body weight (mg/larva) ± SE	LD_{50} (mg *B.t.t.*/larva) x 10^{-6} ± SE
Darmstadt	1.13±0.12 a	2.38±0.12 b
Michelfeld	1.31±0.09 a	2.41±0.53 b

Numbers in a column followed by some letter are not significantly different (P = 0.05) by t-test.

Table 3

Body weight, LD_{50} value and slope for each population after the concentration of all replicates

Population	Body weight (mg/larva) ± SE	LD_{50} (mg *B.t.t.*/larva) x 10^{-6}	LD_{50} 95% fiducial limits	Slope of the regression line ± SE
Darmstadt	1.13±0.12	2.38	2.12–2.68	2.88±0.16
Michelfeld	1.31±0.09	2.12	1.88–2.39	2.08±0.15

Table 4 shows the estimate of relative medium potency with the 95% fiducial limits. By this method the Darmstadt population is just 1.12 times more resistant than the Michelfeld population, but the fiducial limits show that there is no difference between the populations regarding the susceptibility to *B.t.t.*

Table 4

Comparing the *B.t.t.* susceptibility of the populations
(after the concentration of all replicates)

Comparing the population	Estimate of relative medium potency	95% fiducial limits
Darmstadt: Michelfeld	1.12	0.95–1.33

These data do not accord with the results of Whalon et al. (1993), and Rahardja and Whalon (1995). One reason may be that the American scientists have worked under laboratory conditions; the observed resistance of CPB was due to a permanent *B.t.t.* treatment. Another possibility could be that the farm where the Michelfeld population was collected from, is spraying NOVODOR FC only since 1993, once or twice a year. This spraying period may be too short to develop a resistance. The third possibility is that the imagines collected from the farm in Michelfeld were flown in from another region. The probability for this is low, because the farm is relatively separated from others, situated in a valley, and the field of the next potato grower was found 3 km far away, but not 100% excludable.

Although, until now, in our investigations no occurrence of resistance was verified (if NOVODOR FC applied once or twice a year), to avoid the development of resistance, the growers should sometimes alternate NOVODOR FC with other insecticides. However, due to the negative experience in laboratory investigations it will be necessary to test the susceptibility of CPB after every five years in which *B.t.t.* was used, in order to recognize a diminished susceptibility to this pathogen. For these monitoring studies our standardized bioassay method is suitable.

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New Data to the Knowledge of the Aphid Fauna of Hungary (Homoptera: Aphidoidea)

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Author gives a report on the partial results of the regular collections made between 1990 and 1999 on ornamental trees and shrubs, on streets, in parks, in green spaces of housing estates, in private gardens and in arboreta. A total of 45 aphid species were found belonging to 2 families. *Phylloxerina populi* (del Guercio, 1900), *Cinara cedri* Mimeur, 1936, *Neotrama caudata* del Guercio, 1909, *Protrama ranunculi* (del Guercio, 1909), *Aphis catalpae* Mamontova, 1950, *Ovatomyzus boraginacearum* Eastop, 1952 and *Uroleucon taraxaci* (Kaltenbach, 1843) are new for the Hungarian fauna.

Keywords: New aphid species in Hungary, Homoptera, Aphidoidea.

In discovering the aphid fauna in Hungary, works by Horváth (1897), Szalay-Marzsó and Andrásfalvy (1970), Andrásfalvy (1971, 1978), Szelegiewicz (1977), and Szalay-Marzsó (1969, 1989) were significant. Halmágyi (1969, 1974) reported mostly about results of studies on aphid species of forestry tree species, while Haltrich et al. (1992) and Ripka et al. (1993, 1998) about aphid species of ornamental trees and shrubs.

Materials and Methods

Between 1990 and 1999, an aphid survey was made on ornamental trees and shrubs of streets, squares, green areas of housing estates, parks, botanical gardens and private gardens in all districts of Budapest, few samples were collected in some other localities of Hungary and Croatia, also from herbaceous plants. The majority of the results of this survey were reported in the papers of the present author and other co-authors in 1993, 1998. Plant samples from woody plant species (minimum 5 shoots, or 25 leaves/plant) were taken in plastic bags. In Hévíz the aphids were collected with water trap (so-called Moericke yellow trap filled with water plus detergent) by T. Vincze and dr. Z. Ilovai. Hévíz is a famous health resort with warm water not far off the lake Balaton. The water traps were placed on the water level of the lake. During the plant examination with binocular microscope, all the aphids found on the plant samples were put in 70% ethyl alcohol. After boiling the aphids for some minutes in ethyl alcohol they were put in 10% potassium hydroxide. The animals' body were cleaned by boiling them again in it and by pressing them to the necessary extent. Having rinsed the animals with distilled water, they were mounted in Hoyer's medium, containing sorbitol (Keifer, 1975) and in Heinze's medium of polyvinyl alcohol (Schmutterer, 1959). The microscope-slide preparations

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were dried in a thermostat at 32 °C and then sealed with nail varnish. Aphids were studied with phase contrast microscope. In order to identify the aphid species works by Börner (1952), Szelegiewicz (1977), Blackman and Eastop (1984, 1994) were used.

Results

A total of 45 aphid species were found belonging to 2 families *(Table 1). Phylloxerina populi* (del Guercio, 1900), *Cinara cedri* Mimeur, 1936, *Neotrama caudata* del Guercio, 1909, *Protrama ranunculi* (del Guercio, 1909), *Aphis catalpae* Mamontova, 1950, *Ovatomyzus boraginacearum* Eastop, 1952 and *Uroleucon taraxaci* (Kaltenbach, 1843) are new for the Hungarian fauna.

Aphis catalpae caused severe leaf-shrivelling and produced much honeydew on *Catalpa bignonioides. Phylloxerina populi* was found on the bark of branches of *Populus x canescens* associated with *Chionaspis salicis* (Linnaeus) (Homoptera: Coccoidea). The phylloxerid specimens were under the scales of dead diaspidid. Three aphid species were found on roots of weeds. *Anoecia corni* (Fabricius) was collected from roots of *Setaria verticillata, Pemphigus fuscicornis* (Koch) from roots of *Chenopodium album,* and *Neotrama caudata* from roots of *Sonchus oleraceus. P. fuscicornis* was also present on leaves of *Ch. album. Hayhurstia atriplicis* (Linnaeus) was collected from leaves of *Ch. album. Ovatomyzus boraginacearum* was present on underside of leaves of *Geum urbanum.* Colonies of *Uroleucon taraxaci* were found on leaf petioles and flower stalks of *Taraxacum officinale. Rhopalosiphum nymphaeae* (Linnaeus) caused severe infestation on *Nymphaea rubra.*

The following aphid parasitic mite species co-occurred with aphids: *Allothrombium pulvinum* Ewing, *Podothrombium exiguum* Fain et Ripka, *Podothrombium pannonicum* Fain et Ripka, *Charletonia singularis* (Oudemans) and *Erythraeus budapestensis* Fain et Ripka.

Table 1

Aphid species collected in Hungary
(* = new species for the Hungarian fauna)

Aphid species	Plant species/trap type	Sampling place	Sampling date
fam. **Phylloxeridae**			
**Phylloxerina populi* (del Guercio, 1900)			
	Populus x canescens	Óbuda	25.08. 1998
		Óbuda	07.05. 1999
fam. **Aphididae**			
Pemphiginae **Eriosomatini**			
Eriosoma lanuginosum (Hartig, 1839)			
	Ulmus minor	Törökvész	10.06. 1999

Table 1 (cont.)

Aphid species	Plant species/trap type	Sampling place	Sampling date
Pemphiginae **Pemphigini**			
Pemphigus fuscicornis (Koch, 1857)			
	Chenopodium album	Kispest	10.09. 1993
Anoeciinae			
Anoecia corni (Fabricius, 1775)			
	Setaria verticillata	Maglód (Pest c.)	10.09. 1995
Thelaxinae			
Thelaxes suberi (del Guercio, 1911)			
	Quercus cerris	Vászoly (Veszprém c.)	05.07. 1998
Lachninae **Cinarini**			
**Cinara cedri* Mimeur, 1936			
	Cedrus atlantica	Budapest	13.10. 1999
Cinara cupressi (Buckton, 1881)			
	Thuja occidentalis	Békéscsaba (Békés c.)	23.06. 1998
		Budapest	18.10. 1999
Cinara piceae (Panzer, 1801)			
	Picea abies	Pasarét	08.05. 1997
	Picea sp.	Kiskuhalas (Bács-Kiskun c.)	28.04. 1998
Cinara tujafilina (del Guercio, 1909)			
	Juniperus scopulorum	Budapest	14.06. 1999
Lachninae **Lachnini**			
Lachnus roboris (Linnaeus, 1758)			
	Castanea sativa	Érd-parkváros (Pest c.)	20.10. 1997
	Quercus cerris	Vászoly (Veszprém c.)	05.07. 1998
	Quercus robur	Víziváros	03.06. 1997
Lachninae **Tramini**			
**Neotrama caudata* del Guercio, 1909			
	Sonchus oleraceus	Törökvész	17.09. 1993
**Protrama ranunculi* (del Guercio, 1909)			
	Water trap	Hévíz (Zala c.)	25.07. 1997
Chaitophorinae **Chaitophorini**			
Chaitophorus capreae (Mosley, 1841)			
	Salix aegyptiaca	Gellérthegy	02.11. 1999
Chaitophorus sp.			
	Water trap	Hévíz (Zala c.)	16.05. 1997
	Water trap	Hévíz (Zala c.)	30.05. 1997
	Salix aegyptiaca	Pasarét	07.06. 1997
	Salix aegyptiaca	Gellérthegy	02.11. 1999
Periphyllus aceris (Linnaeus, 1761)			
	Acer platanoides	Zirc (Veszprém c.)	26.05. 1997

Table 1 (cont.)

Aphid species	Plant species/trap type	Sampling place	Sampling date
Periphyllus acericola (Walker, 1848)			
	Acer platanoides	Zirc (Veszprém c.)	26.05. 1997
Periphyllus lyropictus (Kessler, 1886)			
	Acer platanoides	Zirc (Veszprém c.)	26.05. 1997
Periphyllus testudinaceus (Fernie, 1852)			
	Acer acuminatilobum	Zirc (Veszprém c.)	26.05. 1997
	Acer platanoides	Zirc (Veszprém c.)	26.05. 1997
Chaitophorinae	**Atherodini**		
Sipha glyceriae (Kaltenbach, 1843)			
	Water trap	Hévíz (Zala c.)	30.05. 1997
Drepanosiphinae	**Phyllaphidini**		
Hoplocallis ruperti Pintera, 1952			
	Quercus cerris	Vászoly (Veszprém c.)	05.07. 1998
Myzocallis komareki (Pasek, 1953)			
	Quercus robur	Széchenyihegy	28.07. 1999
Phyllaphis fagi (Linnaeus, 1767)			
	Water trap	Hévíz (Zala c.)	05.05. 1997
Pterocallis alni (De Geer, 1773)			
	Water trap	Hévíz (Zala c.)	30.05. 1997
Aphidinae	**Pterocommatini**		
Pterocomma rufipes (Hartig, 1841)			
	Water trap	Hévíz (Zala c.)	16.05. 1997
Aphidinae	**Aphidini**		
**Aphis catalpae* Mamontova, 1950			
	Catalpa bignonioides	Víziváros	03.06. 1997
	Catalpa bignonioides	Víziváros	10.09. 1998
Aphis fabae Scopoli, 1763			
	Oxybaphus nyctagineus	Rákoskeresztúr	29.05. 1999
Aphis farinosa Gmelin, 1790			
	Salix aegyptiaca	Pasarét	07.06. 1997
Aphis pomi De Geer, 1773			
	Sorbus torminalis	Kispest	13.06. 1999
Aphis sambuci Linnaeus, 1758			
	Water trap	Hévíz (Zala c.)	06.06. 1997
Rhopalosiphum maidis (Fitch, 1856)			
	Setaria verticillata	Maglód (Pest c.)	10.09. 1995
Rhopalosiphum nymphaeae (Linnaeus, 1761)			
	Nymphaea rubra	Hévíz (Zala c.)	02.04. 1997
	Nymphaea rubra	Hévíz (Zala c.)	09.06. 1997

Table 1 (cont.)

Aphid species	Plant species/trap type	Sampling place	Sampling date
	Sagittaria sagittifolia	Hévíz (Zala c.)	09.06. 1997
	Water trap	Hévíz (Zala c.)	16.05. 1997
	Water trap	Hévíz (Zala c.)	19.06. 1997
Aphidinae	**Macrosiphini**		
Brachycaudus cardui (Linnaeus, 1758)			
	Achillea filipendulina	Törökvész	18.05. 1994
Brachycaudus helichrysi (Kaltenbach, 1843)			
	Water trap	Hévíz (Zala c.)	30.05. 1997
Cavariella aegopodi (Scopoli, 1763)			
	Water trap	Hévíz (Zala c.)	16.05. 1997
Cavariella theobaldi (Gillette et Bragg, 1918)			
	Water trap	Hévíz (Zala c.)	05.05. 1997
	Water trap	Hévíz (Zala c.)	16.05. 1997
	Water trap	Hévíz (Zala c.)	30.05. 1997
Hayhurstia atriplicis (Linnaeus, 1761)			
	Chenopodium album	Maglód (Pest c.)	23.05. 1994
Hyperomyzus lactucae (Linnaeus, 1758)			
	Sonchus oleraceus	Törökvész	25.09. 1993
Hyperomyzus picridis (Börner et Blunck, 1916)			
	Ribes alpinum	Zirc (Veszprém c.)	26.05. 1997
Macrosiphum cholodkovskyi (Mordvilko, 1909)			
	Chenopodium album	Maglód (Pest c.)	23.05. 1994
Macrosiphum euphorbiae (Thomas, 1878)			
	Achillea filipendulina	Törökvész	18.05. 1994
Macrosiphum rosae (Linnaeus, 1758)			
	Rosa sp.	Törökvész	23.10. 1999
Metopolophium dirhodum (Walker, 1849)			
	Rosa sp.	Törökvész	23.10. 1999
**Ovatomyzus boraginacearum* Eastop, 1952			
	Geum urbanum	Törökvész	09.05. 1998
	Geum urbanum	Törökvész	12.05. 1998
Roepkea marchali (Börner, 1931)			
	Prunus mahaleb	Törökvész	13.07. 1998
Uroleucon sonchi (Linnaeus, 1767)			
	Sonchus oleraceus	Maglód (Pest c.)	20.09. 1993
**Uroleucon taraxaci* (Kaltenbach, 1843)			
	Taraxacum officinale	Kispest	29.04. 1995

Discussion

In this survey 14 aphid species were collected from herbaceous plants, 22 species from woody plants, and 10 species used by water trap. At Hévíz the aphids of water neighbouring vegetation were collected.

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New Data to the Knowledge on the Eriophyoid Fauna on Woody Plants in Hungary (Acari: Eriophyoidea)

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Authors give a report on four eriophyoid species which were new for the Hungarian fauna (*Aculops berochensis* Keifer and Delley; *Aculops parakarensis* Bagdasarian; *Aculus ligustri* Keifer; *Aculus? crataegumplicans* Cotte). Three of them were found on new host-plants.

Keywords: Eriophyoid fauna, *Aculops* spp.

The eriophyoid mites are frequent on the cultivated and the adventitious plants, on monocotyledons and dicotyledons. Several researchers' work assisted to know the Hungarian fauna (Balás, 1939; Farkas, 1965, 1966; Dellei and Szendrey, 1989; Győrffy and Molnár, 1992; Ripka, 1997, Ripka and de Lillo, 1997). Farkas (1966) gave a key and general morphological characterization on the 249 species and the 20 subspecies which were found in Hungary until 1966. He described shortly the host-plants and the damages of these pests. Since then the number of the known eriophyoid mites increased considerably. Ripka (1997) observed further 27 new species for the Hungarian fauna and 63 new host-plants.

Materials and Methods

In 1999 many plant samples were taken from various cultivated and adventitious woody plants, from different parts of Hungary. Leaves and sprouts were collected randomly, from each side of the trees and the bushes. The occurrence of eriophyoid species were examined by stereomicroscope in laboratory. All the mites found were put in lactic acid. Lactic acid was used for cleaning, Keifer-II solution for staining of cuticula, and Keifer-III solution for preserving specimens. The identification of the genus was performed by using phase contrast microscope and the key of Boczek et al. (1989). The species were identified by using the catalogue of Davis et al. (1982) and Amrine and Stasny (1994) and on the basis of the original descriptions.

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Results

In 1999 four new species were found in the Hungarian eriophyoid fauna. Three of them appeared on new host-plants *(Table 1).*

Table 1

Eriophyoid mite species collected from woody plants

Species	Host-plant	Sampling place	Sampling date
Aculops berochensis Keifer and Delley, 1971	*Prunus avium**	Kiskunfélegyháza, Bács-Kiskun county	19.02.1999
Aculops parakarensis Bagdasarian, 1972	*Prunus spinosa**	Valkó, Pest county	22.05.1999
Aculus ligustri (Keifer, 1938)	*Ligustrum vulgare*	Budapest	01.06.1999
Aculus? crataegumplicans (Cotte, 1910)	*Crataegus monogyna**	Galgahéviz, Pest county	09.06.1999

*new host-plants

Discussion

Aculops berochensis Keifer and Delley were found on *Prunus avium* in Hungary. Cherry was a new host-plant for this mite. Originally Keifer and Delley (1971) described *Aculops berochensis* on the undersurface of domestic plum *(Prunus domestica)* leaves in Canton of Neuchatel, in Switzerland. According to the catalogue of Amrine and Stasny (1994) this species occurs on *Prunus armeniaca, Prunus cerasus, Prunus domestica, Prunus insititia, Prunus mahaleb, Prunus persica* and *Prunus spinosa.* Castagnoli and Oldfield (1996) listed the following trees as host-plants of *Aculops berochensis:* apricot, mahaleb cherry, sour cherry, European plum, myrobalan plum, peach.

Aculops parakarensis Bagdasarian was collected from *Prunus spinosa* in Hungary. Blackthorn was a new host-plant for this mite. At first Bagdasarian (1972) described *Aculops parakarensis* on the undersurface of almond *(Amygdalus communis)* leaves in Armenia. Amrine and Stasny (1994) and Castagnoli and Oldfield (1996) mentioned the species on *Amygdalus communis* = *Prunus dulcis,* too. In Hungary Ripka (1997) and Ripka and de Lillo (1997) found *Acalitus phloeocoptes* Nalepa, *Phyllocoptes abaenus* Keifer, *Diptacus gigantorhynchus* Nalepa, and *Rhinotergum schestovici* Petanovic on *Prunus spinosa,* but they did not find *Aculops parakarensis.*

Aculus ligustri Keifer was collected on the well-known host-plant of the species, namely on *Ligustrum vulgare,* in Budapest. In 1938 Keifer described this mite and named *Phyllocoptes ligustri* on the above-mentioned plant in California. Later Keifer (1959) changed the name of *Phyllocoptes* to *Aculus.* Ripka (1997) and Ripka and de Lillo (1997) observed *Shevtchenkella ligustri* Farkas on *Ligustrum vulgare* and *Aceria ligustri* Keifer

on the leaves of *Ligustrum ovalifolium,* but did not collect *Aculus ligustri* in Hungary. According to the catalogue of Amrine and Stasny (1994) *Aculus ligustri* can be found on both plants.

Aculus? crataegumplicans Cotte appeared in Hungary as a new species for the Hungarian eriophyoid fauna on *Crataegus monogyna* which was a new host-plant. The species was described by Cotte (1910) on the leaves of *Crataegus oxyacanthoides* and was named *Eriophyes crataegumplicans.* On the basis of Amrine and Stasny (1994) *Aculus? crataegumplicans* occurred on *Crataegus oxyacanthoides* and *Crataegus laevigata.* However, according to Simon (1992) these plants are the same.

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Population Dynamical Stability in Service of Plant Protection Prognosis

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The knowledge of the population dynamical characteristics of the pests fundamentally determines the success of the plant protection prognosis. In this paper we examine the possibilities of the utilization of the information about the population dynamical stability that we get from field examination data. We take into account neighbouring data pairs from data series regarding the change of insect density counted at stated intervals. Using these pairs of values – from the tendency of the absolute and relative changes – we can draw conclusion on the stability of the individual density values or on the stability of the whole dynamical pattern.

Keywords: Population dynamics, plant protection, stability, new methods.

Preparing plant protection prognoses (Manninger, 1968; Benedek et al., 1969, 1974) usually we can give prediction on the changes of the number of individuals based on the actually measured density values of a given pest and with the knowledge of its biological properties (taking into account other factors, too). The knowledge of the phenology and population dynamics of the species comes from the analysis of previous field examination data. In our study we examine the possibilities of the utilization of the information about the population dynamical stability that lies in these data.

Problems and general considerations

Many people in many ways use the expression of population dynamical stability and these meanings can refer to very different phenomena depending on the nature and methods of a survey (May, 1973; Maynard Smith and Slatkin, 1973; Juhász-Nagy and Vida, 1978; Vekerdi, 1978; Huffaker, 1984; Den Boer, 1986). It happens frequently that one part of a phenomenon associated with the stability is named with another specific and more exact definition. In a wider sense by stability we mean the static or dynamic continuity (permanence), survival capability and persistency observable in certain circumstances of the value of a measured, measurable or modelable variable (sometimes variables). The index of stability can vary significantly depending on the circumstances even if we have only one variable. Therefore we often make a distinction between local and global stability. Many times even so is insufficient, in this case we must define more precisely the validity

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interval of the index. An index called "stability" can contain information about very different characteristics of the examined variable. According to the differential equations based on population dynamical models the expression of stability is often used as a special (equilibrium) value of a variable to reveal that once the system was moved away from a given value then was left to itself (disturbance, perturbation) whether it can return to its original value and if so how fast could reach it. Depending on the intensity of disturbance this property is called as global or local resilience too, and the frequently applied linear stability analysis refers also to that. We can measure another, fundamentally different phenomenon regarding the question how big intensity needs an external effect to change the actual value or interval of a variable – this we call resistance. The expression of robust we can consider as a generalization of resistance, when we examine in how wide range of a factor (or factors) could be invariable the given value (or interval) of a selected variable.

We can use very well the preceding and many other similar expressions in case of mathematical models or well arranged laboratory experiments, and in this way we can approach (make easier) to understand the phenomena of nature. However in the plant protection prognosis the field examination data have a great importance too, what is more, mainly these form the basis of the practical methods (Benedek et al., 1974; Nowinszky, 1994, 1997). Analyzing the field data (obtained e.g. by light-, color- or sex traps) we can observe states of equilibrium very rarely concerning the measured density values, moreover the interpretation of these is realizable only in special cases. However, in case of the most pests we have very few information, often only hypothesis about the factors which have effect on the abundancy of the population. As a starting point we have only the population dynamical pattern detectable in the time changes of density values. Looking at the curves about the changes in the number of individuals it is inevitable to have a subjective impression about their stability character. If the number of individuals vary rapidly in a wide range without any predictable way, we feel that variable less stable than another with slow, gradual, little by little changes. If we replace this subjective impression with a numeric expression, the information content of the data becomes utilizable. To this we only need to give the actual definition of stability and the method of measurement.

Recommended expressions of stability

In case of great number of data we can interpret the stability in two ways:
– concerning individual density values or intervals
– concerning the whole density pattern.

Let us consider absolutely stabile that density value in which proximity the population changes with only few individuals by unit of time and relatively stabile where with small rate. For this reason by stability of the individual density values we mean the reciprocal of the size of absolute and relative changes.

The population dynamical pattern we can consider as absolutely stabile when with the increment of the density values the absolute stability values belonging them also increase and instabile when they decrease. The field data are practically always instabile in this respect, so in this case the rate of the decrease and its slowing, constant or accelerating characteristic provides information about the stability. We can consider the

pattern as relatively stabile when with the increment of the density values their relative stability also increase and instabile when it decreases. In some cases with the increment of the density values the stability values change in a not monotonous way, in these cases we can describe the stability of the whole pattern giving the stability character for each density interval.

Recommended investigation methods

- We can start from the number of individuals caught at stated intervals (or other observed density values). In case of continuous observation we can make the analysis using different time-scale summations too, but we can compare only anlyses prepared in the same way. At the analysis of the possible scale dependence it is practical to take into consideration the cycles by generations, years etc.
- From the neighbouring values we form pairs.
- We measure the absolute changes with the difference N2–N1, where we substract always the former value from the following one independently of the size.
- To express the relative change we calculate the ratio 2*(N2–N1)/(N2+N1). This indicates how does the absolute change between two times (or periods) compare with the average density during this change ((N2+N1)/2).
- We can calculate these indices for all data pairs and represent them in the following manners:
  a) absolute changes against the time
  b) relative changes against the time
  c) absolute changes against the average number of individuals
  d) relative changes against the average number of individuals
  e) absolute and relative changes against each other.

The **a** and **b** type figures are suitable to describe more precisely the phenological events and to interpret the relationship of generations. In the present study we do not deal with these. The figures **c** and **d** make visible the fact and mode of density dependence. The **e** type figure helps to understand more exactly the phenomena. The figures **c, d** and **e** afford possibility not only to compare with one another (in all combination) the phenomena observed in different species, areas or years but to compare them directly using mathematical models, that was impossible till now using only the basic data. From the **c** and **d** type figures, obtained from the processing of great number of data, we can directly read (in case of the dynamics manifested in the figure) what could be the maximal extent of the change by unit of time after a given density value (number of individuals or frequency of individuals (density) – depending on the applied method). In our opinion this information (although it does not show the direction of change) could have an important role also in the practice of plant protection prognosis.

Processing appropriate number of data splitted by different time scales we can get such population dynamical analyses that promote the biological cognition of the species exceeding the phenological characteristics. Completing all these analyses for many species we have the possibility to classify the species based on the characteristics mentioned above, and so we can approach to the explanation of phenomena related to population dynamics.

We can express the absolute and relative instability belonging to the given density values (or intervals) with the range or standard deviation of the adequate deviation values.

The stability character of the whole pattern we can read directly in the **c** and **d** type figures or in the representation of the values given before.

Case studies

To illustrate the conception presented above we present three dynamical patterns. In all cases we use two pairs of **c** and **d** type figures, with values calculated from real data and from the result of a model with similar behaviour to them. The y axis of the **c** type figures shows the absolute changes, the y axis of the **d** type figures shows the absolute changes. The x axis represents always the average number of individuals (it means the density values during the changing period). The data series are based on real field experiments, but we selected them on purpose to represent well the different simple basic patterns. In reality the majority of data series does not have such a clear-sighted form but they are mixtures of several patterns like these. The characteristic of a pattern may vary in time and space, on the other hand it is scale dependent. Making comparisons we must take all these into consideration. The presented models are used for emphasize the essentials, in themselves they are unsuitable for prediction. The patterns of the models show the behaviour of the extreme values from the pattern of the field data and these are what we examine also in case of field data, because these are of great importance in practical aspect, too. In the models the dynamics of increase and decrease is always symmetrical and usually this is the situation in reality too, but it is not necessary. Preparing analyses in order to become acquainted with the species we need much more data pairs than marked in these figures that calls the attention to the importance of the LTER examinations. All three examples are based on data from the national light trap system, data of 10 areas with "hot summer" summarized by ten days.

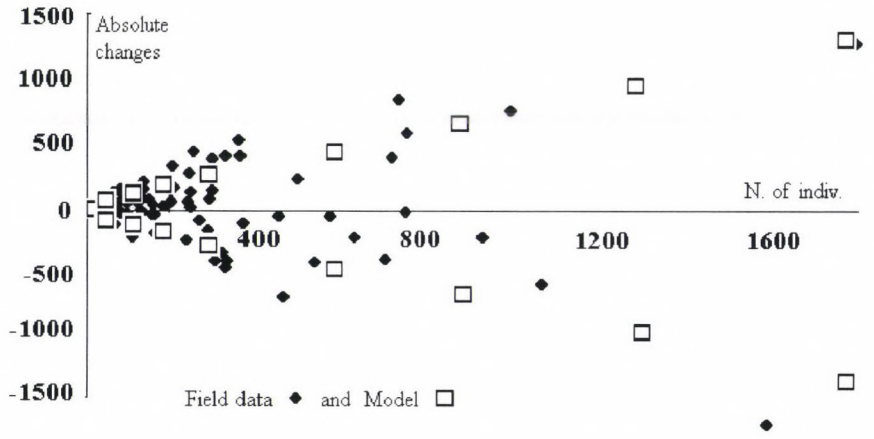


Fig. 1.1. Generations of *Sitochroa verticalis* between 1964 and 1969

Example 1
Generations of *Sitochroa verticalis* between 1964 and 1969

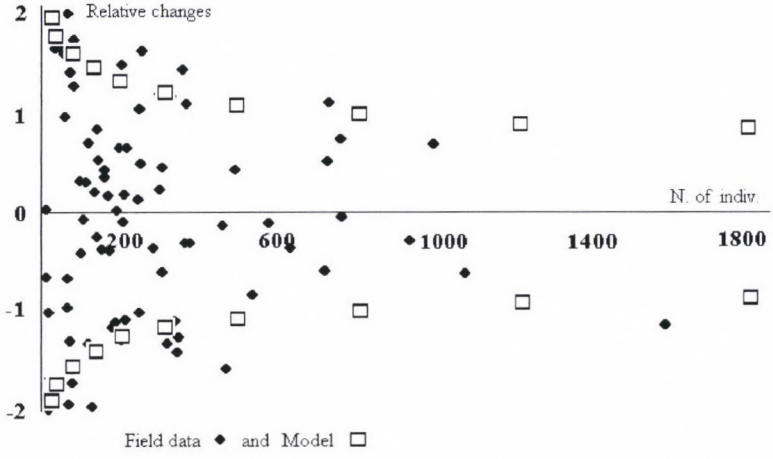


Fig. 1.2. Generations of *Sitochroa verticalis* between 1964 and 1969

The extreme values against the density follow a slowing increase regarding the absolute changes and slowing decrease regarding the relative ones. This behaviour is similar to models with the *n*th power, where $0<n<1$ *(Figs. 1.1* and *1.2)*

Example 2
The second non-gradational generations of *Evergestis extimalis* between 1964 and 1969
Concerning the absolute changes the extreme values increase until a maximal density and then they decrease, the relative changes have an accelerating increase.
The applied model: $N_t = N_{t-1} + aN_{t-1} - bN_{t-1}^c$, where $a>1$, $0<b<1$, $c>1$.
This is similar to a logistic model *(Figs. 2.1* and *2.2).*

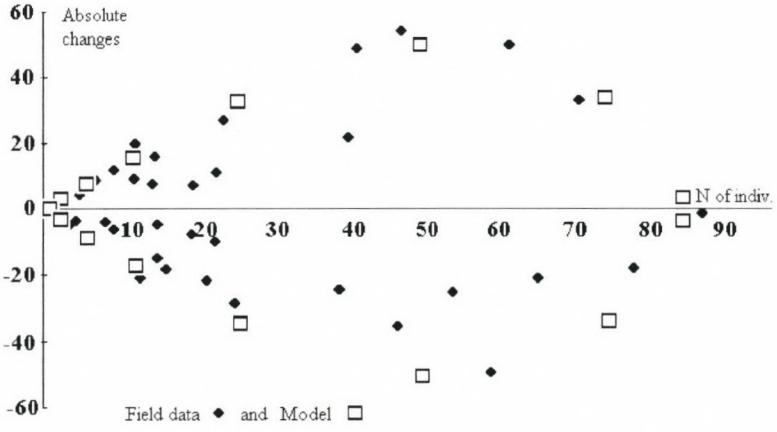


Fig. 2.1. The second non-gradational generations of *Evergestis extimalis* between 1964 and 1969

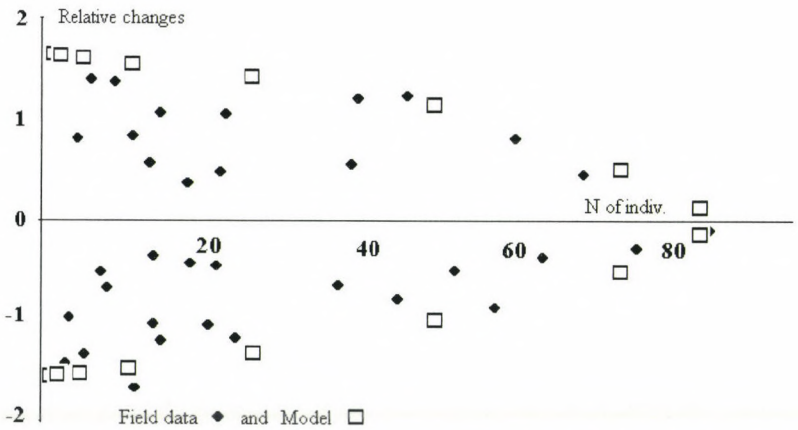
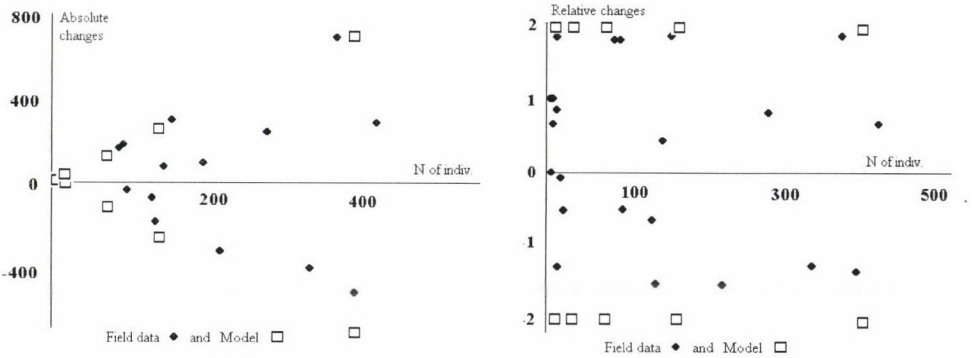


Fig. 2.2. The second non-gradational generations of *Evergestis extimalis* between 1964 and 1969

Example 3
The second generations of *Etiella zinkenella* between 1963 and 1969
Concerning the absolute changes the extreme values have a linear increase, the relative changes show a constant form.
The applied model: $N_t = N_{t-1} + aN_{t-1}$, where $a>1$.
This is similar to the exponential model *(Figs. 3.1* and *3.2).*



Figs 3.1 and 3.2. The second generations of *Etiella zinkenella* between 1963 and 1969

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MAGYAR
TUDOMÁNYOS AKADÉMIA
KÖNYVTÁRA

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Methodological Investigations on a Neuropteroidea Community

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The same Neuropteroidea community was collected and studied in Hungary in the years 1991 and 1992 by using different trapping techniques: a) Malaise trap, b) suction trap, c) yellow pan trap and d) light trap. The studies aimed to compare the different sampling methods for individual species, families and for the whole Neuropteroidea community.

In case the whole Neuropteroidea community the trapped individual numbers collected by the suction trap surpassed all other trap types. Relatively high numbers of Neuropteroidea could be collected both by light trap and Malaise traps. The yellow pan traps did not succeed in catching large enough samples neither from point of view of sample size nor from species richness. According to the number of species collected there were not discovered any big difference between the catches of suction trap, Malaise traps and light trap.

By evaluating according to families it was stated that in case of the family *Raphidiidae* the Malaise trap yielded larger and more diverse samples than any other methods; however by increasing the number of yellow pan traps it is possible to augment the number of caught individuals. The suction traps were found very satisfactory in collecting members of the family *Coniopterygidae*. For the members of the families *Hemerobiidae* and *Chrysopidae* both the suction traps and light traps were found effective, although suction traps were more successful in collecting *Chrysopidae* species.

Similarly, by evaluating the data according to individual species it was found that the Malaise traps tended to "under-represent" species belonging to *Hemerobius humulinus, Hemerobius Lutescens, Chrysopa pallens* and the ones belonging to *Chrysoperla carnea* complex compared to the suction trap. The opposing situation was perceived with *Micromus lanosus* and *Chrysopa perla*. Similarly – compared to suction trap – the light trap significantly "under-represented" the species *Hemerobius humulinus, Hemerobius lutescens, Chrysopa pallens, Chrysoperla carnea* and *Dichochrysa prasina* and "over-represented" *Micromus angulatus, Sympherobius pygmaeus* and *Chrysopa phyllochroma*.

The diversity of Neuropteroidea collected by Malaise trap and light trap surpassed significantly the one of suction trap and yellow pan traps. The assemblages collected by different sampling methods showed some overlapping, but differed in their characters.

Keywords: *Raphidiidae, Coniopterygidae, Hemerobiidae, Chrysopidae,* sampling techniques, Malaise trap, suction trap, light trap, yellow pan trap.

Many ingenious instruments have been devised that are successful in catching large quantities of insect taxonomical groups but only a few of these are suited for quantitative insect sampling (Muirhead-Thomson, 1991). A theoretically good sampling technique has to represent the structure of a community without deformations. From technical point of view our instruments have been brought gradually near to perfection, but in spite of this it still has to be said that our quantitative collections often reflect only the minimal values of the majority of the species occurring in the ecosystem (Balogh, 1953).

Looking over the investigations on the Neuropteroidea assemblages either in natural or in agricultural ecosystems it seems that from methodological point of view this group has not been studied with the same intensity as some other groups like *Coleoptera*

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and *Lepidoptera* (Muirhead-Thomson, 1991). To the community surveys, sweeping nets, light traps, Malaise traps, pan traps, sticky traps, McPhail traps and suction traps were used and these assessments were carried out mostly against adults (Banks, 1952; Canard et al., 1979; Greve and Andersen, 1975; New, 1967; Szabó and Szentkirályi, 1981; Szentkirályi, 1992; Sziráki, 1996).

Materials and Methods

Our studies have carried out in 1991 and 1992 in the experimental orchard of the Plant Protection Institute of the Hungarian Academy of Sciences near Budapest (Nagykovácsi, Juliannna-major, North latitude: 47° 30' and East longitude 19°; UTM: CT47). The mixed, abandoned orchard with secondary succession lies in the Buda Hills on 470 m height above the sea level, surrounded by oak forests *(Quercetum petreae-cerris* and *Ceraso-mahaleb-quercetum pubescentis)* and ruderal vegetation. The two-hectare orchard containing different fruit species and varieties was established in 1967 and had not received since 20 years any plant protection treatments. Earlier faunistical studies were carried out in the orchard by Szabó and Szentkirályi (1981).

One Meszlény and Szalay-Marzsó (1979) type suction trap was placed on the edge of the orchard, near to peach trees. The engine of the trap operated on 220 V (55W) and had a capacity of 1000 m^3 airflow per hour. The mouth of the aspirator was 48 cm in diameter and worked in a height of 160 cm. The trap was emptied twice daily, in the morning and evening twilight.

Two white coloured Thownes-type Malaise traps (Townes, 1962; Móczár, 1967) were used, one in the centre of the orchard, 40 m distance from the edge, the other on the edge of the orchard near of an oak forest, 20 m distance from the suction trap. The height of the traps was 3.5 m with catching area of 10 m^2.

One Jermy type (Jermy, 1961) light trap was used 40 m distance from the edge, not too far from the second Malaise trap. It was attached 1.8 m above the ground and operated with 100 W lamp and emptied daily in 1991. The *Coniopterygidae* from the material collected by light trap were not separated.

Five yellow pan traps were placed into the orchard at height 1.5 m. Two pans were placed near the suction trap, and one near all the other type traps. Each trap (14 cm x 20 cm x 10 cm) was halfway filled with 30% ethylene glycol in water. The yellow pan traps and Malaise traps were emptied three times a week. All the traps operated from mid-March until mid-November in both years.

In the characterisation of the *Neuroptera* assemblages the characters of species richness and diversity are generally used. As the different diversity indexes show well-known different sensitivities towards the extremes: common and rare species, the lacewing assemblages were characterised by diversity ordering function, where a scale parameter (α) underwent continuous increasing. As a result we obtained the profile of diversity of the Neuropteroidea community from the low (sensitivity to rare species) to high (sensitivity to common species) scale parameters. For this diversity arrangement the one used by Rényi (1961) was chosen (Tóthmérész, 1995).

$$H\alpha = \frac{\log \sum_{i=1}^{S} p_i^{\alpha}}{1 - \alpha}$$

where $\alpha = 0, \alpha \neq 1$
S: Total number of species
p_i: Percentage of species i in the total assemblage

Comparison of the samples of four different trap types and of two distinct years was performed by the program Syntax 5.1 using multivariate data analysis methods, namely hierarchical classification (complete linkage) and ordination (principal coordinates analysis – PCoA). Both the cluster analysis and the PCoA methods were based on Horn index (Krebs, 1989).

Results

In course of our survey 1849 individuals of 43 *Neuroptera* species were collected in total *(Table 1)*. Considering the individual numbers the highest values were brought by the suction trap, followed by the light trap and Malaise traps *(Tables 1* and *2)*. However, if the family *Coniopterygidae* is excluded, there are no significant differences between the number of species collected by the three methods *(Tables 1* and *2)*. The collecting successes of the five yellow pan traps were left behind the other trap types.

Table 1

Individual densities of *Neuroptera* species in function of different collecting methods applied. Nagykovácsi, 1991 and 1992

	Suction trap		Malaise traps[1]		Yellow pan traps[2]		Light trap[3]
	1991	1992	1991	1992	1991	1992	1991
Raphidiidae							
Dichrostigma flavipes Stein	2	2	21	11	12	2	0
Raphidia ophiopsis Linnaeus	0	0	0	2	1	0	0
Subilla confinis Stephens	0	0	0	7	0	0	0
Xanthostigma xanthostigma Schummel	0	0	9	11	0	0	0
Inocellidae							
Inocellia crassicornis Schummel	0	0	1	0	0	0	0
Parainocellia braueri Albarda	0	0	1	0	0	0	0
Coniopterygidae							
Coniopteryx arcuata Kis	46	18	0	0	0	0	–
Coniopteryx borealis Tjeder	4	19	0	0	0	0	–
Coniopteryx esbenpeterseni Tjeder	54	150	0	0	0	0	–
Coniopteryx haematica McLachlan	0	3	0	0	0	0	–
Coniopteryx lentiae Aspöck et Aspöck	0	1	0	0	0	0	–

Table 1 (cont.)

	Suction trap		Malaise traps[1]		Yellow pan traps[2]		Light trap[3]
	1991	1992	1991	1992	1991	1992	1991
Coniopteryx pygmaea Enderlein	0	1	0	0	0	0	–
Coniopteryx renate Rausch et Aspöck	7	31	0	0	0	0	–
Coniopteryx tineiformis Curtis	4	21	0	0	0	0	–
Helicoconis pseudolutea Ohm	4	13	0	0	0	0	–
Parasemidalis fuscipennis Reuter	0	1	0	0	0	0	–
Semidalis aleyrodiformis Stephens	18	33	0	0	0	0	–
Hemerobiidae							
Hemerobius humulinus Linnaeus	17	5	7	1	2	0	1
Hemerobius lutescens Fabricius	35	17	10	2	0	0	14
Hemerobius micans Olivier	0	5	0	3	0	0	0
Micromus angulatus Stephens	0	0	2	4	0	0	23
Micromus lanosus Zeleny	0	0	4	5	0	0	3
Micromus variegatus Fabricius	0	0	0	1	0	0	2
Sympherobius elegans Stephens	0	0	1	0	0	0	0
Sympherobius pygmaeus Rambur	1	4	3	0	0	0	10
Wesmaelius betulinus Strom	0	0	0	0	0	0	6
Wesmaelius subnebulosus Stephens	1	1	2	0	1	0	1
Chrysopidae							
Chrysopa dorsalis Burmeister	0	2	0	0	0	0	0
Chrysopa formosa Brauer	15	67	7	15	0	0	19
Chrysopa nigricostata Brauer	0	3	0	0	0	0	0
Chrysopa pallens Rambur	30	47	0	6	1	1	0
Chrysopa perla Linnaeus	6	21	28	72	5	9	24
Chrysopa phyllochroma Wesmael	1	0	0	0	0	0	30
Chrysopa viridana Schneider	0	12	0	0	0	0	0
Chrysopa walkeri McLachlan	0	0	1	0	0	0	2
Chrysoperla carnea Stephens	187	287	11	13	31	2	41
Chrysotropia ciliata Wesmael	2	0	0	0	0	0	0
Dichochrysa flavifrons Brauer	2	7	0	0	1	0	0
Dichochrysa prasina Burmeister	37	57	13	17	5	1	1
Hypochrysa elegans	0	0	0	1	0	0	0
Nineta flava Scopoli	1	0	0	0	0	0	0
Nothochrysa fulviceps Stephens	1	2	0	0	0	0	0
Peyerimhoffina gracilis Schneider	0	1	0	0	0	0	0
Abundance (without *Coniopterygidae*)	338	540	121	171	59	15	177
Number of species	22	28	16	16	9	5	14

[1]in case of Malaise traps, the data of two traps are pooled
[2]in case of yellow pan traps the data of five traps are pooled
[3]in case of light trap catches the species of *Coniopterygidae* family were not considered.

Table 2

Number of *Neuroptera* species and individual densities in function of different sampling methods applied. Nagykovácsi, 1991 and 1992

	Suction trap		Malaise traps[1]		Yellow pan traps[2]		Light trap[3]
	1991	1992	1991	1992	1991	1992	1991
No. of species							
Raphidiidae	1	1	2	4	2	1	0
Inocellidae	0	0	2	0	0	0	0
Coniopterygidae	7	11	0	0	0	0	–
Hemerobiidae	4	5	7	6	2	0	8
Chrysopidae	10	11	5	5	5	4	6
No. of individuals							
Raphidiidae	2	2	30	31	13	2	0
Inocellidae	0	0	2	0	0	0	0
Coniopterygidae	137	291	0	0	0	0	–
Hemerobiidae	54	32	29	16	3	0	60
Chrysopidae	282	506	60	124	43	13	117

[1]in case of Malaise traps, the data of two traps are pooled

[2]in case of yellow pan traps the data of five traps are pooled

[3]in case of light trap catches the species of *Coniopterygidae* family were not considered.

It is remarkable that species belonging to the family *Coniopterygidae* were present only in the suction trap *(Table 2, Fig. 1)* compared to the Malaise traps and yellow pan traps.

The *Raphidiidae* were significantly underestimated by the suction trap compared to the Malaise traps and the yellow pan traps *(Table 2, Fig. 1).* The *Raphidiidae* material caught in the Malaise and pan traps were considerable; both as regards the collected number of individuals and in case of Malaise trap the species number (4 species). In the light trap Raphidioptera species did not occur *(Table 2).*

By studying the family *Hemerobiidae* it can be stated that neither the light trap nor the suction trap did not show significant differences. The sizes of samples collected by the two Malaise traps fell significantly behind the two latter methods. The best results were shown by the light trap as far as the species numbers are concerned (8 species). The two Malaise traps (7 and 6, respectively, in course of the two years 9 species in total) were found more effective than the suction trap (4 and 5, respectively, in course of two years 5 *Hemerobiidae* species in total). The yellow pan traps were not found suitable to collect species of *Hemerobiidae (Table 2).*

The suction trap surpassed the other trapping methods as members of the *Chrysopidae* family are concerned. For individual numbers the suction traps were followed by the light traps, Malaise traps and yellow pan traps *(Table 2, Fig. 1).* The suction trap was also superior to other methods as far as the numbers of species were collected. The Malaise traps yielded samples similar to light traps and exceeded the species richness of yellow pan traps only by few species. The yearly fluctuations of individual numbers were the highest in yellow traps *(Table 2).*

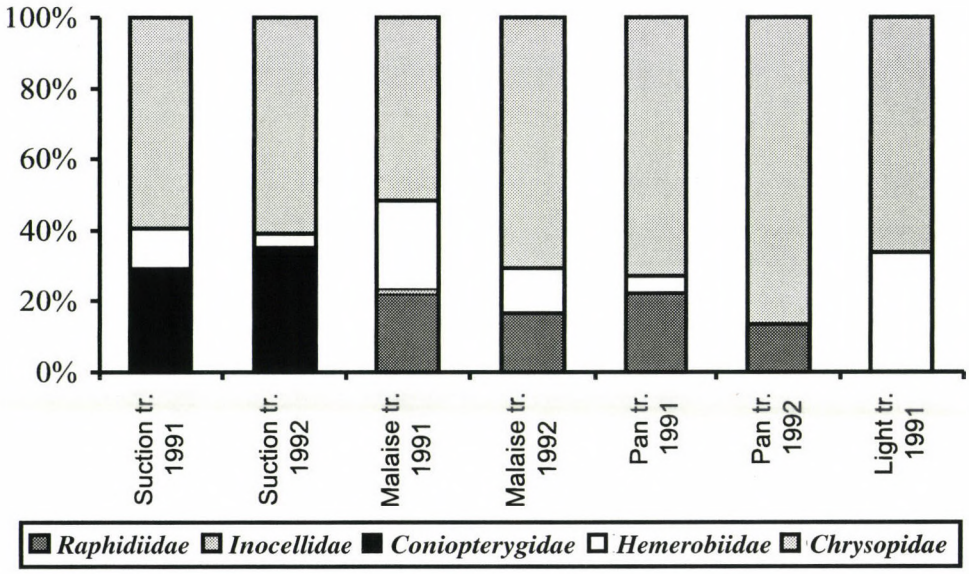


Fig. 1. Dominance of different families, plotted against different collecting methods (From the material collected by light traps the species of *Coniopterygidae* were not selected out)

By looking separately the individual species of *Raphidiidae* and *Coniopterygidae* we find that conclusions established for families are also valid for species. The members belonging to *Raphidiidae* were collected to a higher degree by Malaise traps compared to suction trap and light traps whereas Malaise traps and yellow pan traps did not contain members of the *Coniopterygidae* family *(Table 1).*

Within the family *Hemerobiidae* the species *Hemerobius lutescens* was the most frequent in the suction traps in both years, while the Malaise and light traps showed its relative frequency much lower *(Table 3). Micromus lanosus* was not collected by suction traps in either year. In the light trap *Hemerobius lanosus* was under-represented while *Micromus angulatus* and, to a smaller extent, *Sympherobius pygmaeus* was over-represented compared to other trap types, especially to suction traps *(Tables 1* and *3).*

Within the family *Chrysopidae,* the species *Chrysopa formosa* was collected by all trap types with similar frequency *(Table 3).* The suction trap over-represented the species *Chrysopa pallens* and *Chrysoperla carnea,* as compared to other trap types. The light trap over-represented *Chrysopa phyllochroma* and under-represented *Dychchrysa prasina.* In Malaise traps the individual numbers of *Chrysopa perla* were significantly over-represented, while *Chrysoperla carnea* was under-represented, compared to other trap types *(Tables 1* and *3).* The yellow pan traps collected the species *Chrysopa perla, Chrysoperla carnea* and *Dichochrysa prasina* with higher frequencies, although the total individual number stayed quite low and the results of different years were very dissimilar. Although results of yellow pan traps were not shown in *Table 3,* it could be established that

Table 3

Relative densities of *Hemerobiidae* and *Chrysopidae* species, in function of different sampling methods applied. Those that showed higher densities are shown by grey colour (total *Hemerobiidae* = 1, total *Chrysopidae* = 1)

	Suction tr. 1991	Suction tr. 1992	Malaise tr. 1991	Malaise tr. 1992	Light tr. 1991
Hemerobiidae					
Hemerobius humulinus	0.31	0.16	0.24	0.06	0.02
Hemerobius lutescens	0.65	0.53	0.34	0.12	0.23
Micromus lanosus	0	0	0.13	0.31	0.05
Micromus angulatus	0	0	0.06	0.25	0.38
Sympherobius pygmaeus	0.02	0.13	0.10	0	0.17
Chrysopidae					
Chrysopa formosa	0.05	0.13	0.12	0.12	0.16
Chrysopa pallens	0.11	0.09	0	0.04	0
Chrysopa perla	0.02	0.04	0.47	0.57	0.21
Chrysopa phyllochroma	0	0	0	0	0.26
Chrysoperla carnea	0.66	0.57	0.18	0.1	0.35
Dichochrysa prasina	0.13	0.11	0.22	0.13	0.01

the traps did not under-represented *Chrysopa perla* ($p_{i1991} = 0.12$ and $p_{i1992} = 0.69$ – when the total number of *Chrysopidae* collected in 1991 or in 1992 is 1.0) compared to suction traps and *Dichochrysa prasina* ($p_{i1991} = 0.12$ and $p_{i1992} = 0.08$) as compared to the light trap.

If the data of suction traps are regarded as the least distorted ones – owing to the fact that this trap types do not exert any attractive influence on flying insects and only the ones just happen to fly over the trap are sucked – in by accident – we can establish that in Malaise traps compared to suction traps the species *Hemerobius humulinus, Hemerobius lutescens, Chrysopa pallens* and *Chrysoperla carnea* were significantly under-represented *(Table 3),* while *Micromus lanosus* and *Chrysopa perla* were heavily over-represented. Similarly, the light traps significantly under-represented the species *Hemerobius humulinus, Hemerobius lutescens, Chrysopa pallens* and *Chrysoperla carnea* and *Dichochrysa prasina* and over-represented the species *Micromus angulatus, Sympherobius pygmaeus* and *Chrysopa phyllochroma (Table 3).*

By studying the diversity conditions of lacewing assemblages by using Rényi-diversity and by excluding *Coniopterygidae* it was established that the diversities of samples got by suction trap and yellow pan trap are especially lower than the ones collected by Malaise trap and light trap *(Figs 2* and *3, Tables 4* and *5).* The diversities of *Neuroptera* assemblages collected by Malaise and light traps showed significant differences only at the begin of scale parameter, in the section sensitive to rare species, contrary to other sections of the scale parameter *(Figs 2* and *3, Tables 4* and *5).*

If by evaluating the diversities of suction trap catches also the species belonging to *Coniopterygidae* were considered, then the diversity of Neuropteroidea assemblage had increased and only on that section of the scale parameter fell behind the diversity of

Malaise trap and light trap catch where it was sensitive to frequent or medium frequent species (1991) or the scale parameter in its whole length did not differ from the diversity shown by the Malaise trap (1992).

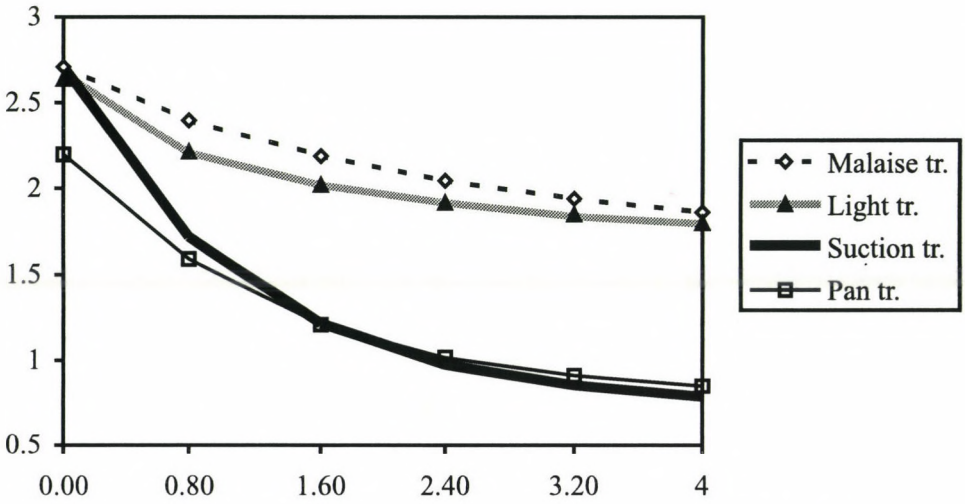


Fig. 2. Rényi-diversity profiles of *Neuroptera* assemblages collected by different methods. Nagykovácsi, 1991 (The *Coniopterygidae* were excluded from the sampling data of suction traps)

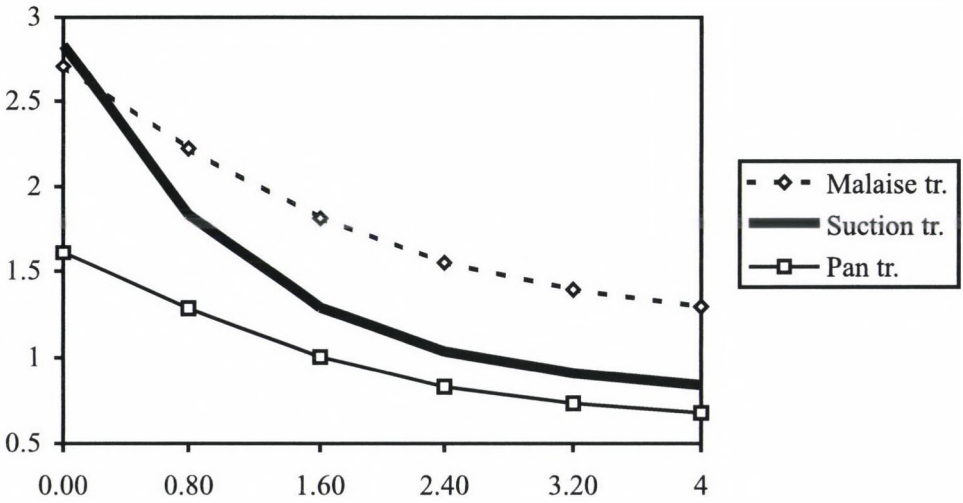


Fig. 3. Rényi-diversity profiles of *Neuroptera* assemblages collected by different methods. Nagykovácsi, 1992 (The *Coniopterygidae* were excluded from the sampling data of suction traps)

The similarities of Neuropteroidea assemblages observed by different trapping methods were studied with the Horn index. Both the hierarchical classification *(Fig. 4)* and ordination *(Fig. 5)* showed significant segregation in the samples collected by suction, Malaise and light traps. The assemblages collected by yellow pan traps did not exhibit definite character: in 1991 the collected material resembled the one of suction trap, in 1992 more to the one found in Malaise traps.

Table 4

Comparison of Rényi diversities of *Neuroptera* assemblages collected by different methods in 1991 by t-test ($^{*}p < 0.05$, $^{**}p < 0.01$, n.s.= non-significant, t-values)

	0.8	1.6	2.4	3.2	4
Suction tr. – Malaise tr.	** 7.2267	** 7.5233	** 6.8563	** 6.1441	** 5.6064
Suction tr. – Pan tr.	n.s. 0.9381	n.s. 0.0385	n.s. 0.2263	n.s. 0.3279	n.s. 0.3660
Suction tr. – Light tr.	** 5.9745	** 8.1984	** 8.9013	** 8.8250	** 8.3787
Malaise tr. – Pan tr.	** 5.6825	** 5.1661	** 4.8272	** 4.4932	** 4.2343
Malaise tr. – Light tr.	* 2.0776	n.s. 1.3584	n.s. 0.8087	n.s. 0.4867	n.s. 0.3039
Pan tr. – Light tr.	** 4.6233	** 4.7707	** 5.0174	** 5.0965	** 5.0742

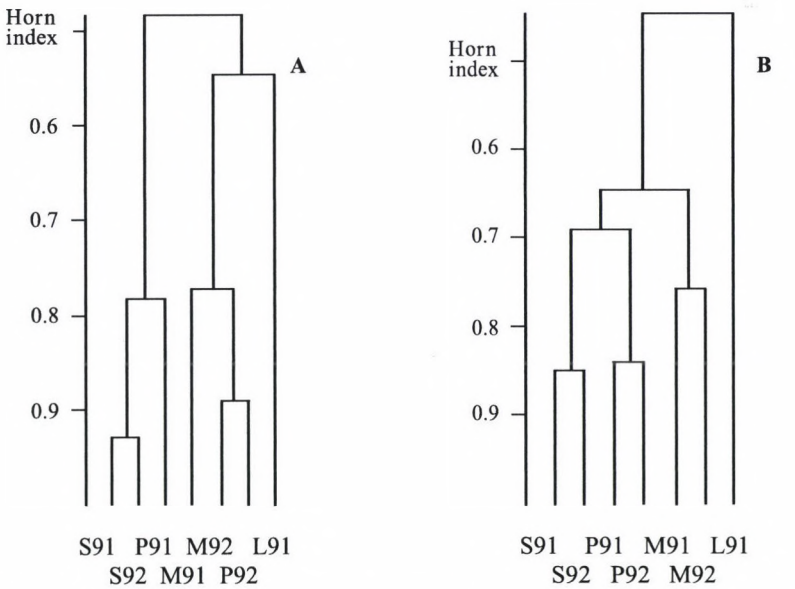


Fig. 4. The similarities of *Neuroptera* assemblages collected by different trapping methods and compared by hierarchical clustering (Nagykovácsi, 1991–1992). A: Complete link, Horn index B: the same, but with standardisation ($\log_{10}$)
(The *Coniopterygidae* were excluded from the sampling data of suction trap)

Table 5

Comparision of Rényi diversities of *Neuroptera* assemblages collected by different methods in 1992 by t-tests, Nagykovácsi
(+: P< 0.1 , *: p< 0.05, **: p< 0.01, n.s.: non-significant; and t-values)

	0.8	1.6	2.4	3.2	4
Suction tr. – Malaise tr.	** 3.5426	** –3.1649	** 2.7124	* 2.5991	* 2.4271
Suction tr. – Pan tr.	* 2.8347	n.s. 0.9502	n.s. 0.6565	n.s. 0.5899	n.s. 0.5721
Malaise tr. – Pan tr.	** 4.4417	* 2.4481	+ 2.0215	+ 1.9008	+ 1.8628

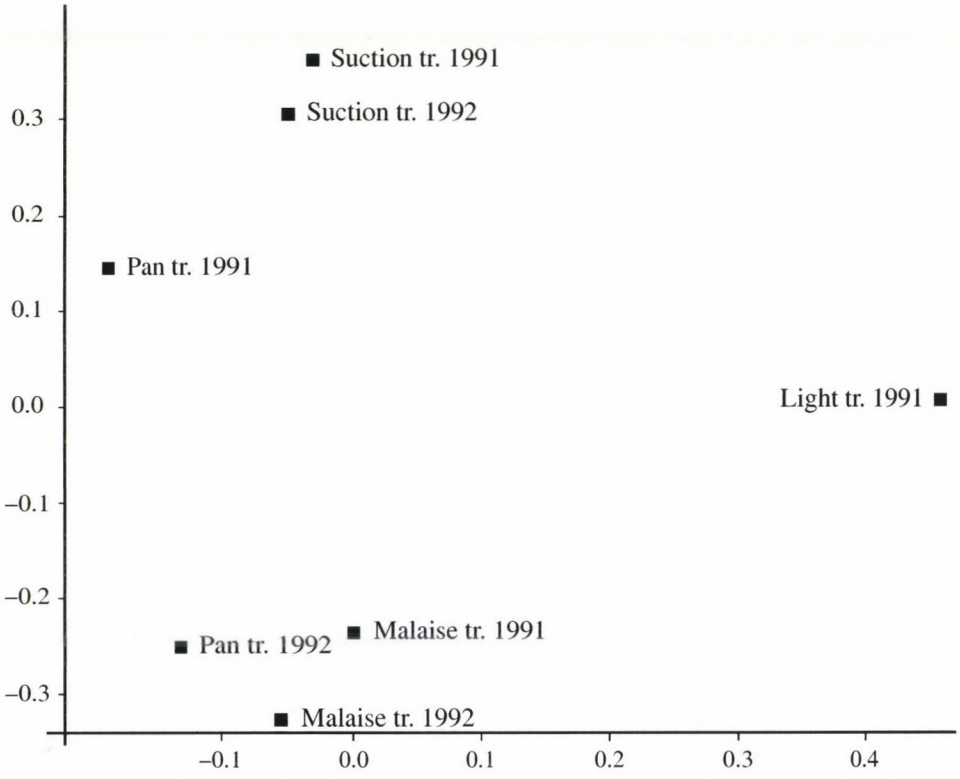


Fig. 5. Comparison of *Neuroptera* assemblages collected by different methods and evaluated by metric ordination (method of principal coordinates analysis, Horn index). Nagykovácsi, 1991–1992
(The *Coniopterygidae* were excluded from the sampling data of suction trap)
S 91 and S 92: suction traps in 1991 and 1992
M 91 and M 92: Malaise traps in 1991 and 1992
P 91 and P 92: pan traps in 1991 and 1992
L 91: light trap in 1991

Discussion

According to our observations the collection of *Raphidioptera* assemblage is carried out mainly by Malaise traps or to a smaller extent by yellow pan traps, wich phenomenon may be in connection with their diurnal activity. For the collection of *Coniopterygidae* we have found the most suitable the suction traps while Malaise traps and yellow pans did not collect species belonging to this family. The results in case of Malaise traps are inconsistent with the experiences of Vidlicka (1994, 1995) who found in his Malaise traps more or less *Coniopterygidae.* In our case the diameter of guiding-wing (baffle) meshes of traps were wide enough (2 millimetres) for the trapped insects to escape. Although the *Coniopterygidae* of light traps were not singled out, this does not mean that they are not attracted by light. According to the literature (Williams and Killington, 1935) *Coniopterygidae* are well collected by light traps and we have observed the same in other habitats. From *Hemerobiidae* the light traps caught the majority of species. This trap type was recommended by Szabó and Szentkirályi (1981) and Szentkirályi (1992) to collect *Hemerobiidae.* In *Chrysopidae* the suction trap yielded 2.5 times more individuals than the light traps and 6–8 times more than the two Malaise traps; even the number of species was higher in case of the suction trap. Our results contradicted the ones of Szabó and Szentkirályi (1981) who found the best the light traps in collecting *Chrysopidae* in other habitats.

The Malaise and light traps yielded significantly distorted results and samples compared both to each other and to ones of suction traps. For example the Malaise traps over-represented the presence of *Micromus lanosus,* the opposite was true in case of *Hemerobius* species. At the same time the light trap over-emphasised the presence of *Chrysopa phyllochroma* compared to the other traps. It results from this that the structure of the samples are remarkably determined by the interactions between the species composition of the natural Neuropteroidea assemblages and the sampling method have been used.

Although there were significant fluctuations within populations in different years, these were equalised on assemblage level by using the same trap type; if however different traps were used, the significant differences caused separations in Neuropteran assemblages. The results of Malaise and suction traps kept constantly segregated from each other and from the assemblages collected by the light trap.

The latter (i. e. distortion of the light trap) results from the fundamental differences that these traps work during the dark phase, so the results are influenced by moonlight and by differences of various species in their attraction to light. While most species studied by us show a nocturnal activity (Duelli, 1986; Ábrahám and Vas, 1999; Vas et al., 1999), according to Bowden (1981) moonlight has decreased significantly the flying activity of *Neuroptera.* So the flight activity measured at full moon was not decreased only by the distorting effect of light traps used. A further effect may be caused by the insects themselves: a) the two sexes may be attracted by light to a different degree or b) the bad flyers do not reach the traps but make a landing earlier.

In case of Malaise traps, their functioning in absence of electricity and the high number of species attracted are unquestionable advantages that justified their general use.

At the same time, the large white catching surfaces may exert influence towards different Neuropteroidea species. A further difference may be caused by behavioural differences that result from the behaviour of the insect by landing on the baffles (guiding surfaces). Our observations indicated that after landing the insects walk upward on these.

Although the yellow colour may be attractive to Neuropteroids (Maredia et al., 1992) the whole catching surface may be – even in case of many yellow pan traps – too small to yield large samples. This explains the fact that in our studies the yellow traps gave the smallest samples both in species numbers and individual numbers. However other distortions are possible: besides the mentioned visual effects it has to be mentioned that the yellow pans operate only during the daytime and also the liquid in the pans can influence the insects during the landing process.

Summarised, we can establish that the used methods determine fundamentally the picture showing up on Neuropteroidea assemblages. Plotted against the different methods used we may obtain different values on the relative frequencies, dominance orders, diversities and similarities of assemblages. Even if in the course of following studies the parallel collecting methods often cannot be applied, our results can help to choose the most adequate methods or to interpret correctly the results given by different sampling techniques.

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Study of Neuropteroidea (Raphidioptera, Neuroptera) Communities by Using Malaise Traps in an Untreated Orchard and its Environment

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Neuropteroidea communities were studied near Budapest (Nagykovácsi) in an abandoned, mixed orchard and its neighbouring environment: a shrub community without a closed canopy; a shrub level of the canopied oak forest by using Malaise traps.

In the open shrub verge of the orchard larger, and in the oak forest more diverse Neuropteroidea community developed than in the other investigated habitats.

The Neuropteroidea communities studied did not show stable compositions in the investigated habitats and years.

By studying the linkage conditions of different species to different habitats, it was established that *Hypochrysa elegans* was strongly bound to the shrub level of the closed oak forest. Besides that, both in the shrub level and in the open forest edge, the species *Micromus lanosus, Micromus angulatus, Hemerobius micans, Hemerobius lutescens* and *Hemerobius humulinus* were found with higher density. The species *Dichrostigma flavipes, Chrysopa formosa, Chrysopa perla, Xanthostigma xanthostigma, Chrysoperla carnea* and *Dichochrysa prasina* occurred in all three habitats, thus they have to be regarded as habitat generalists. By passing beyond these tendencies *Dichrostigma flavipes, Chrysopa formosa* and *Chrysopa perla* seemed to be more attached to the shrubby edge.

Keywords: Raphidioptera, Neuroptera, habitat preference, orchard, oak forest.

Many papers have dealt with the habitat preference of Neuroptera (Killington, 1936; Aspöck et al., 1980; Duelli, 1980a, 1980b; Altieri and Todd, 1981; Zeleny, 1984; Szentkirályi, 1989, 1992a). By considering the importance of study of Neuropteroidea communities, investigations were started to detect, which factors can be either

a) internal, determined by physiological and genetical causes like feeding habits, readiness for dispersion (Duelli, 1980a, 1980b) and

b) external, biotic and abiotic environmental factors (Honek, 1981). A part of these external factors determined the composition of the surrounding vegetation and connected phytophagous elements, as potential prey animals. In natural, diverse habitats large neuropteroid communities, which contain many species, can be formed (Honek, 1981; Monserrat and Martín, 1994, 1996; Sziráki, 1996). In agro-ecosystems (both in hard- and soft wooded ones) generally poorer neuropteroid communities, that contained however large individual numbers and whose continuous, gradual growth resulted from the natural capacity of edge habitats to produce diverse species, have been found (Szabó and Szentkirályi, 1981; Szentkirályi, 1989, 1992b; Paulian and Andriescu, 1996).

The authors of the present paper wanted to study this edge effect in the surrounding of mixed, non-cultivated orchard and wanted to obtain answers to the following questions:

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– which species and in what individual densities of neuropteroids occur in different habitats;

– what are the similarities and differences in the compositions of neuropteroid assemblages occurring in different habitats;

– how closely are different species connected to different habitats.

In the course of our studies we used Malaise traps that are especially suitable to collect flying insects, although they are mostly recommended to catch Diptera and Hymenoptera species. Móczár (1967) and Roberts (1970) considered the Malaise traps useful in determining the characteristic species of insect faunas and in giving a comprehensive view on insect communities – due to their uninterrupted, day and night functioning. Juillet (1963), Móczár (1967), Moed and Heads (1987) in their observations found the number of neuropteroids rather limited, whereas Vidlicka (1994, 1995) found these traps suitable for collecting Neuropteroidea.

Materials and Methods

Our studies have been carried out at the 2-hectare abandoned mixed orchard of the Research Institute of Plant Protection of the Hungarian Academy of Sciences near Budapest, in Nagykovácsi (Júlianna-major). Ten years before the beginning of present study also the chemical weed control of the orchard was terminated and discontinued.

In the untreated plantations and in their surroundings three Townes type Malaise traps were installed. As all the three belonged to the same type, it was supposed that their effects in distorting the catching result were also similar, therefore negligible in the comparisons. The traps used were made of a white 2.0-millimetre mesh, so they were unsuitable for collecting Coniopterygids. The catching surface of the trap wings amounted to 10 m^2 in total, the one of the cylinder jacket 15 m^2.

The first trap was placed into the middle of the orchard, into an open spacing between two rows of pear and prune trees; the second was set into the 30 m wide open space between the plantation and the neighbouring oak forest, that was covered by diverse low shrubs. The third trap was set at a distance of 100 m into the mixed, canopied oak forest *(Querceto-petreae-cerris* and *Ceraso petreae pubescentis).* The traps were operated in 1991 and 1992 from mid-April until early October and were emptied three times weekly.

In the course of processing the data of the collected material the diversity values were calculated by using the Rényi diversity formula (RTS) (Tóthmérész, 1995); the similarities were calculated by metric ordination, by principal co-ordinate analysis (PCoA) and by using the Horn and Jaccard indexes.

Results

In course of two years 410 individuals of 25 species were collected in total *(Table 1).* The Neuropteroidea communities showing the highest abundance were formed on the edges; 1.5–2 times higher than in the oak forest and in the orchard *(Table 1, Fig. 1).*

Considering the number of species, the richest community appeared in the forest and on the edge whereas the lowest species number was observed in the orchard itself in both study years *(Table 1, Fig. 1)*.

Table 1

Number of different Neuropteroidea species collected in an untreated, mixed orchard, neighbouring open, bushy edge and a shrub level of an oak forest Nagykovácsi, Hungary, 1991–1992, Malaise trapping

Species	Forest 1991	Edge 1991	Orchard 1991	Forest 1992	Edge 1992	Orchard 1992
Chrysopa formosa Brauer	1	7	0	4	10	5
Chrysopa pallens Rambur	0	0	0	0	4	2
Chrysopa perla Linnaeus	2	13	15	9	58	14
Chrysopa walkeri McLachlan	0	0	1	0	0	0
Chrysoperla carnea Stephens	1	7	4	8	4	9
Cunctochrysa albolineata Killington	0	0	0	2	0	0
Dichochrysa flavifrons Brauer	0	0	0	1	0	0
Dichochrysa prasina Burmeister	3	7	6	13	3	14
Dichrostigma flavipes Stein	12	18	3	0	7	4
Hemerobius humulinus Linnaeus	2	7	0	3	1	0
Hemerobius lutescens Fabricius	5	10	0	2	2	0
Hemerobius micans Olivier	1	0	0	6	2	1
Hypochrysa elegans Burmeister	2	0	0	7	1	0
Inocellia crassicornis Schummel	0	0	1	0	0	0
Micromus angulatus Stephens	5	2	0	2	2	2
Micromus lanosus Zeleny	4	4	0	9	4	1
Micromus variegatus Fabricius	0	0	0	0	0	1
Nineta flava Scopoli	0	0	0	1	0	0
Parainocellia braueri Albarda	0	0	1	0	0	0
Raphidia ophiopsis Linnaeus	0	0	0	2	0	2
Subilla confinis Stephens	0	0	0	1	3	4
Sympherobius elegans Stephens	1	1	0	0	0	0
Sympherobius pygmaeus Rambur	0	3	0	0	0	0
Wesmaelius subnebulosus Stephens	0	2	0	0	0	0
Xanthostigma xanthostigma Schummel	4	5	4	0	4	7
Number of individuals	43	86	35	70	105	66
Number of species	13	13	8	15	14	13

Table 2

Comparison of the Rényi diversities of the Neuropteroidea assemblages of the different studied habitats at different scale parameters by t-test

	1	2	3	4	5	6
Forest – Edge	4.28 **	5.24 **	5.78 **	5.87 **	5.69 **	5.42 **
Forest – Orchard	4.09 **	4.42 **	4.19 **	3.88 **	3.61 **	3.39 **
Edge – Orchard	0.33 n.s.	0.68 n.s.	1.19 n.s.	1.39 n.s.	1.45 n.s.	1.46 n.s.

t values; ** $p < 0.001$, n.s. – non-significant

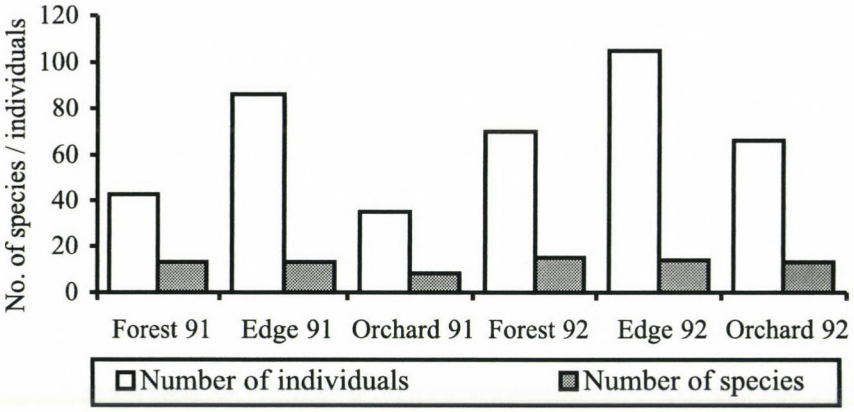


Fig. 1. Development of density and species richness of Neuropteroidea communities in an untreated, mixed orchard, neighbouring open, bushy edge and a closed, canopied oak forest

By studying the diversities and by drawing together the data of the two years it was established that the community appearing at the shrub level of the oak forest has been more balanced and for the whole length of the scale parameter more diverse than the Neuropteroidea communities of other habitats *(Fig. 2)*. The differences between the forest and the edge were significant in the total length of the scale parameter *(Table 2, Fig. 2)*.

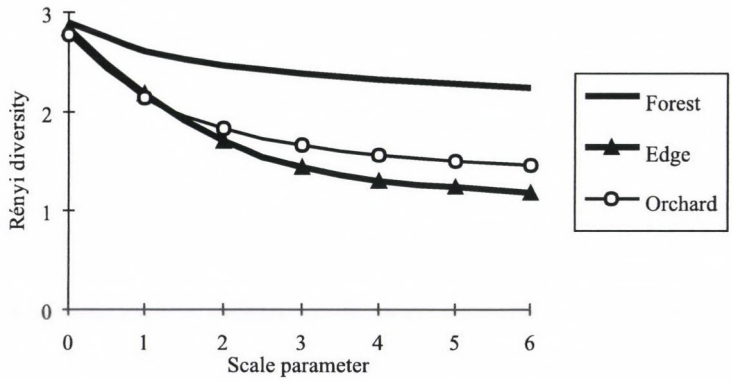


Fig. 2. Rényi diversities of neuropteroid communities, collected by Malaise traps in an abandoned, mixed orchard, neighbouring open, bushy edge and in the shrub level of an oak forest, Nagykovácsi, Hungary, 1991–1992

By studying the similarities (Horn index) it has been established that in different habitats and different years and considering the species composition and also the dominance conditions, no really characteristic communities have been segregated *(Fig. 3)*. The comparison by Jaccard index showed similar results.

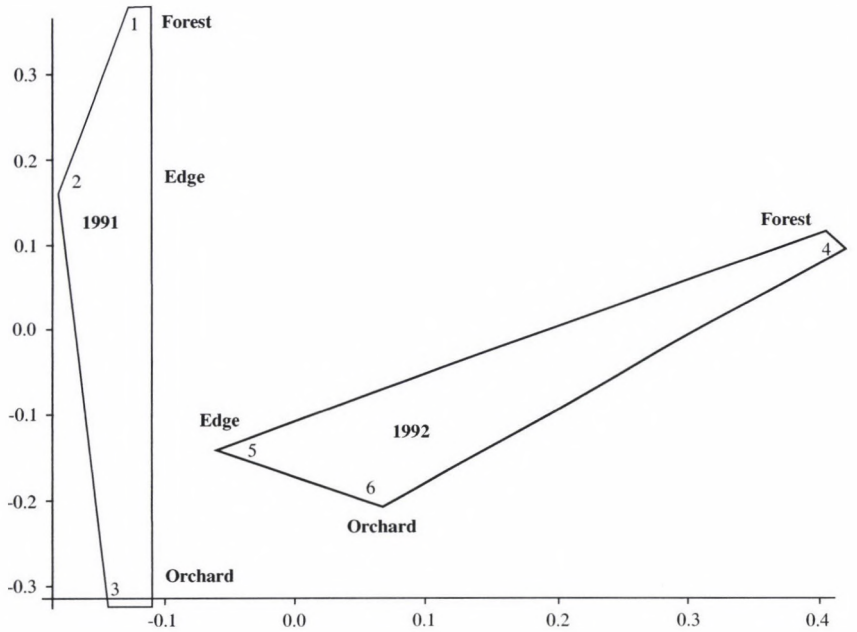


Fig. 3. Study of similarities of Neuropteroidea communities that appeared in different habitats (Malaise trapping), Nagykovácsi 1991–1992. PCoA method, Horn similarity

By exploring the individual Neuropteroidea species *(Table 3)* it was found that *Hypochrysa elegans* was strongly bound to the shrub level of closed oak forests while *Micromus lanosus, Micromus angulatus, Hemerobius micans, Hemerobius lutescens* and

Table 3

Linkage of different Neuropteroidea species to the shrub level of a canopied, closed oak forest, an untreated, abandoned mixed orchard and to an open, diverse, bushy edge between them; individual numbers. Malaise trapping, Nagykovácsi, 1991–1992

Species	Forest	Edge	Orchard
Hypochrysa elegans	9	1	0
Micromus lanosus	13	8	1
Micromus angulatus	7	4	2
Hemerobius micans	7	2	1
Hemerobius lutescens	7	12	0
Hemerobius humulinus	5	8	0
Dichrostigma flavipes	12	25	7
Chrysopa formosa	5	17	5
Chrysopa perla	11	71	29
Xanthostigma xanthostigma	4	9	11
Chrysoperla carnea	9	11	13
Dichochrysa prasina	16	10	20

Hemerobius humulinus occurred both in the forest shrub level and in the open edges. The species *Dichrostigma flavipes, Chrysopa formosa, Chrysopa perla, Xanthostigma xanthostigma, Chrysoperla carnea* and *Dichochrysa prasina* can be regarded as biotope generalists.

Passing beyond these tendencies it can be stated that *Dichrostigma flavipes, Chrysopa formosa* and *Chrysopa perla* attained their highest densities in open, diverse edge vegetation, although they occurred in other habitats as well.

Discussion

In our examinations of open forest edge, covered with diverse vegetation, interspersed with brushes the Neuropteroidea communities were larger and contained more species than the ones in the untreated orchard. This higher density resulted partly from the high numbers of Hemerobiids bound to oak forests and of other species that are linked to edge habitats, like *Dichrostigma flavipes, Chrysopa formosa, Chrysopa perla.* Higher species diversity was found in the shrub level of the oak forest and in the diverse brushland of the edge, however the highest diversity was found in the shrub level of the close canopied forest. Our results well fitted into the conclusions drawn by Szentkirályi (1989, 1992a, 1992b) in his reports on the Neuropteroidea of maize fields and Hemerobiidae of apple plantations. According to his data – in the vicinity of more diverse habitats – both the species number and diversity of communities increased. After abandoning the orchard the diversity of Neuropteroidea communities increased and became similar to the Neuropteroidea communities of the edge. However the species richness did not reach the ones of the other two habitats. The picture became further differentiated by our observations on the diversity of verge communities. The diversity of Neuropteroidea in the shrub level of closed, dense forests was significantly higher than the one of orchards. The scale parameters even surpassed the diversity values of mediocre and common species, compared with the values of forest edges.

As regards the similarities of communities it was concluded that in the different years and habitats no stable communities are formed; i.e. the composition of the Neuropteroidea communities are defined basically – besides the characteristics of their typical habitat – by other factors. It seems that in different years the environments are able to evolve Neuropteroidea communities of stable dominance order only in case of extremely homogenous surrounding vegetation (maize monoculture) and low diversity (Szentkirályi, 1989).

Our data concerning the preference of neuropteroid species to different habitats conform well to some results of Zeleny (1984). *C. perla* and *C. formosa* occurred along the edges of forests and woods, and *C. carnea* and *Dichochrysa prasina* were reported as common in oak forests and forest edges and also in orchards (Zeleny, 1984).

Acknowledgements

We have to thank László Kazinczy and Mrs. L. Hornok for the maintenance of traps, Dr. Ferenc Szentkirályi for his valuable remarks made by reading our manuscript. The study itself has been supported by the OTKA Foundation (No. F23885).

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Revision of *Newsteadia* (Homoptera: Coccoidea) of the Nearctic and Neotropic Regions, with Descriptions of New Species

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Six new species of *Newsteadia* are described from the Nearctic and Neotropic Regions. A key is presented for the identification of 10 species currently included in the genus from these regions. The number of species in the genus worldwide is 37. A modified concept of the genus is presented and some new characters are given as diagnostic of the genus. The zoogeography of the genus is discussed.

Keywords: Homoptera, Coccoidea, Ortheziidae, *Newsteadia,* Nearctic and Neotropic Regions, new species.

The genus *Newsteadia* was studied in detail by Morrison (1925, 1952). Since 1952 one new species *(N. trisegmentalis)* was described from the Nearctic Region (USA, Ga) by Howell (1975). Only one species, *N. tristani* (Morrison, 1952) was recorded from the Neotropic Region (Costa Rica, and Mexico). New distribution records in the Eastern USA, of the two known species *(N. americana,* and *N. minima)* are given in the work of Kosztarab (1996). There are no records of this genus in the western part of USA (Morrison, 1952; Gill, 1993; Miller et al., 1999).

The purpose of the present work is to provide a revision of the genus *Newsteadia* in the Nearctic and Neotropic Regions, including descriptions of some new species.

Materials and Methods

The insects described in this study were collected mostly using a Berlese funnel and are from the Hungarian collecting expeditions of soil arthropods (more than 1000 samples), from 12 countries (Chile, Cuba, Santa Lucia, Brazil, Mexico, Falkland Islands, Ecuador, Paraguay, Costa Rica, Bolivia, Peru, Venezuela) (Dr. János Balogh, Collection of Arachnida, University of Loránd Eötvös (ELTE), Dr. Sándor Mahunka, Collection of Arachnida, Hungarian Natural History Museum, Budapest, Hungary) (HNHM). The collection numbers in the text are from the collection of Dr. J. Balogh and Dr. S. Mahunka. In these samples 168 contained specimens of Ortheziidae, among them 39 samples contained *Newsteadia* species, 121 specimens of Mixorthezia, 8 specimens of Orthezia, and none of Nipponorthezia. The specimens of previously described species are in the collection of the US National Collection of Insects, Beltsville, Maryland, USA (USNM), and University of Napoli (Portici).

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The insects studied were mounted on microscope slides, or partly preserved in alcohol. The holotypes of new species are deposited in the Hungarian Natural History Museum, Budapest (HNHM). Paratypes are deposited in the Plant Protection Institute, Hungarian Academy of Science (PPI), and in the collection of USNM (Beltsville, USA).

Terminology follows that of Morrison (1925, 1952), and of Miller and Kozár (1999, in preparation) except for some new characters found in the present work.

Results

Newsteadia americana Morrison, 1925

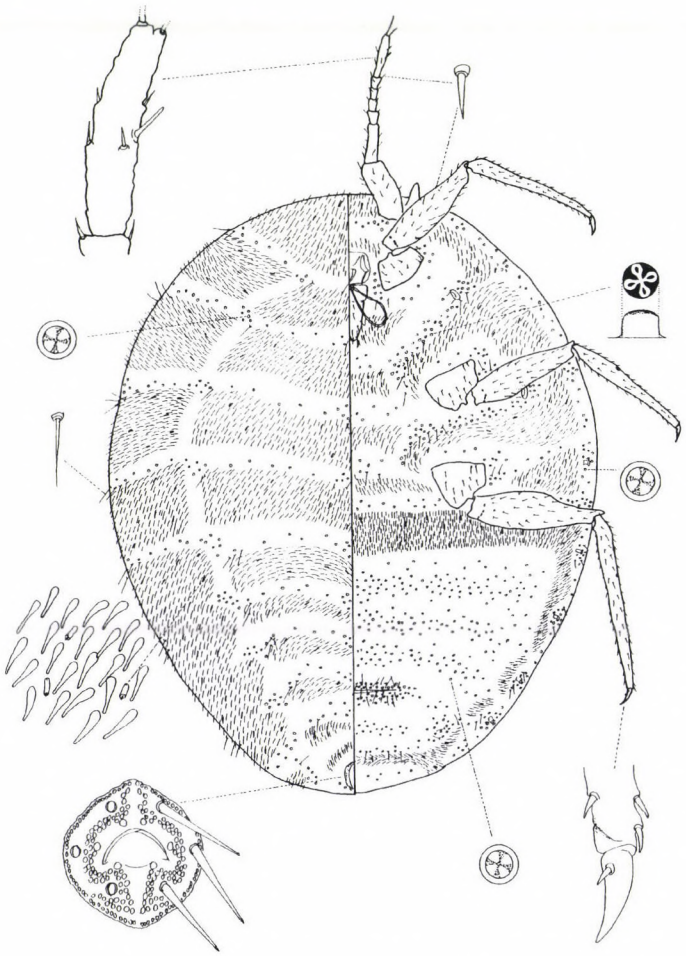


Fig. 1. *N. americana* Morrison, 1925, female

MATERIAL EXAMINED

Holotype: female, USA, Maryland, 08. 08, 1917, Leg: H. S. Barber, on the same slide one paratype. Two paratype females on one slide, USA, Virginia shore, opp. Plummers Is, Md, June 15, 1922, Leg: H. Morrison. One paratype female, Canada, Ontario, Grimsby, Leg. Horn.

The species is different from all species known in the Region by densely covered body with wax plates including the venter of the thorax. A new drawing is presented *(Fig. 1),* because the original drawing does not contain several important characters, like the number of quadrilocular pores on the venter of abdomen and other parts of the body.

This species was not found in our samples.

Newsteadia borhidii sp. n.

MATERIAL EXAMINED

Holotype: female, Cuba, Havana, 16. 02. 1989, Leg: F. Mészáros, No 238. Paratype: two females on one slide, Cuba Sierra Esperon, Leg: A. Borhidi, No. 243, and 1 female, Brazil, Fazenda Aqua Azul, 1967, Leg: J. Balogh, No. B 24.

DESCRIPTION OF ADULT FEMALE

Mounted specimen *(Fig. 2):* Holotype adult female 1.2 mm long, 0.8 mm wide. Antennae 3-segemented, 1st segment 186 μm long, 2nd segment 102 μm long, apical segment 390 μm long, all segments with clavate setae, the longest is 47 μm long, with 9 setae on apical segment, apical seta 114 μm long, without subapical seta, medial sensory seta 34 μm long situated near end of segment.

VENTER

Labium 166 μm long, partly with clavate setae. Stylet loop as long as labium. Legs: 1st coxa 90 μm long, trochanter-femur 320, tibia-tarsus 432 μm long, claw 42, claw digitules destroyed, 2nd coxa 94 μm long, trochanter-femur 390, tibia-tarsus 457, claw 41, claw digitules destroyed, 3rd coxa 98 μm long, trochanter-femur 415, tibia-tarsus 498 μm long, claw 43, claw digitules 7 μm long, with clavate setae from coxae to middle of tibia-tarsus and with spinelike setae on posterior part of tibia-tarsus; without flagellate sensory setae near tibial sensorium on all legs; trochanter with 4 sensory pores on each surface. Ovisac band short, not interrupted in middle, with a special depression covered by quadrilocular pores in front of middle part of ovisac, ovisac wax plate groups on last segments of abdomen absent; wax plates almost absent on the thorax and head. Coxal depressions not developed. Anterior thoracic spiracles without quadrilocular pores, anterior thoracic spiracles 26 μm in diameter. Hairlike setae scattered in medial areas of thorax. Quadrilocular and simple pores 4–7 μm in diameter; tubular quadrilocular pores long, protruding from derm, associated with wax plates; quadriloculars and simple pores present in wide bands within ovisac band and in groups between wax plates elsewhere. Abdominal spiracles five pairs.

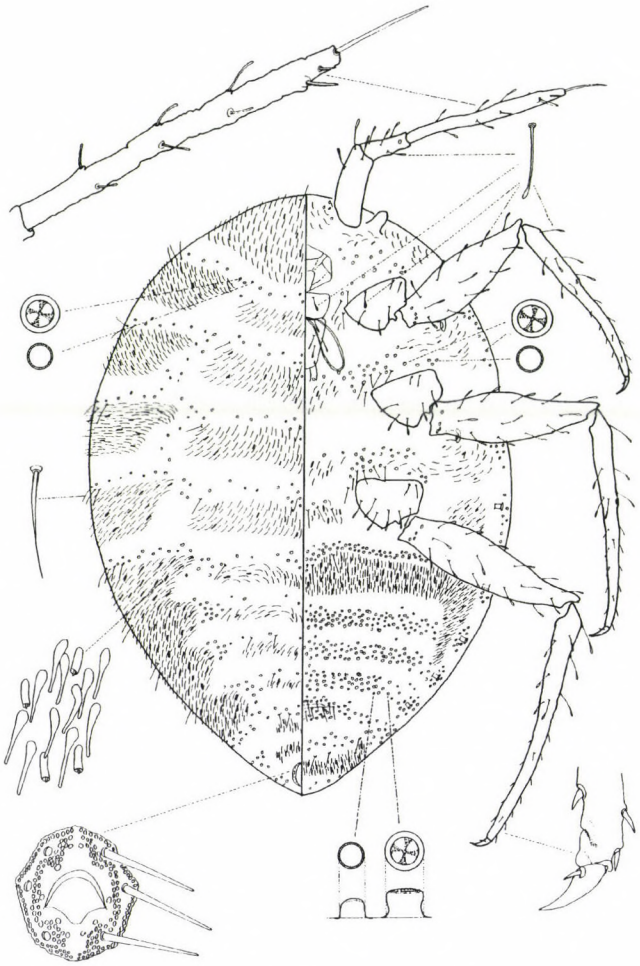


Fig. 2. *N. borhidii* sp. n., female

DORSUM

Wax plates cover only part of surface. Spines at margin of wax plates elongate, 18 μm long. Hairlike setae present in marginal clusters near posterior edges of marginal wax plates, and near anterolateral and anteromedial edge of each dorsomedial wax plate, 37 μm long. Quadrilocular and discoidal pores scattered between wax plates, 8 μm long tubular ducts present. Anal ring with rows of pores; longest anal ring seta 42 μm long, anal ring 48 μm wide and 65 μm long.

The species is named after the collector Dr. Attila Borhidi, Hungarian botanist, who worked for many years in Cuba and collected several interesting scale insect species in different parts of the world.

COMMENTS

N. borhidii is similar to *N. tristani,* but differs by having clavate setae on all segments of the antennae, and by the presence of long tubular ducts.

Newsteadia brasiliensis sp. n.

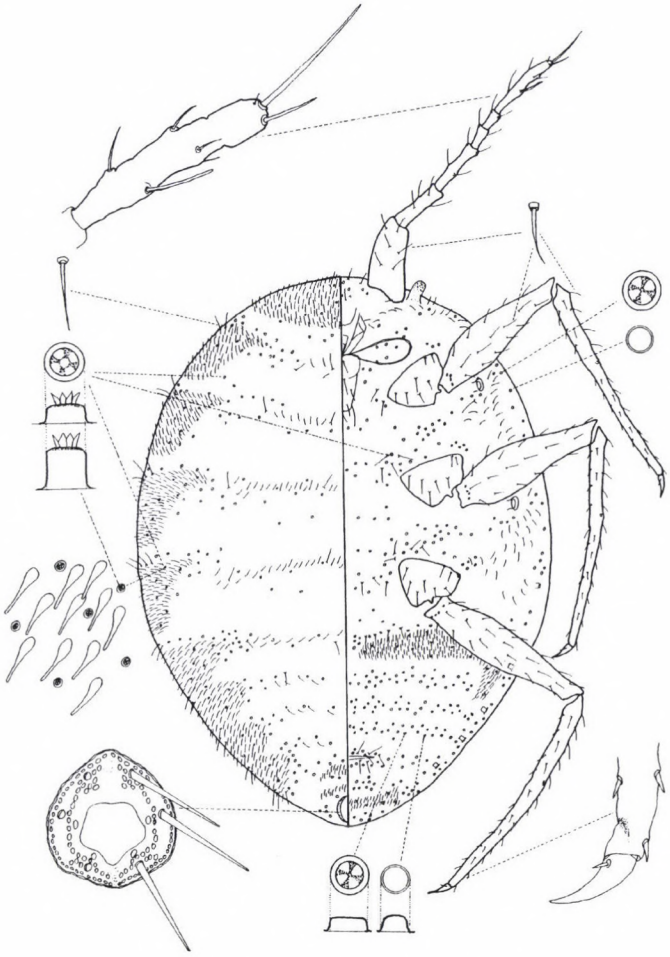


Fig. 3. *N. brasiliensis* sp. n., female

MATERIAL EXAMINED

Holotype: female, Brazil (1550 m a.s.l.), 1995, Leg: J. Balogh, No. B 2. Paratypes: one female from the same collection as the holotype.

DESCRIPTION OF ADULT FEMALE

Mounted specimen *(Fig. 3):* Holotype adult female 1.0 mm long, 0.8 mm wide. Antennae 6-segmented, 1st segment 186 μm long, 2nd segment 122 μm long, apical segment 141 μm long, with 24 μm long, strong hairlike setae, with 7 setae on apical segment, apical seta 94 μm long, with one, 34 μm long subapical seta, medial sensory seta 42 μm.

VENTER

Labium 110 μm long. Stylet loop as long as labium. Legs: 1st coxa 77 μm long, trochanter-femur 320, tibia-tarsus 440 μm long, claw36, claw digitules 7 μm long, 2nd coxa 84 μm long, trochanter-femur 326, tibia-tarsus 465, claw 37, claw digitules 7 μm long, 3rd coxa 96 μm long, trochanter-femur 352, tibia-tarsus 498 μm long, claw 40, claw digitules 7 μm long, partly with strong hairlike, and spinelike setae; flagellate sensory setae near tibial sensorium absent on all legs; trochanter with 4 sensory pores on each surface. Ovisac band interrupted in middle, short, ovisac wax plate groups on last segments of abdomen absent; wax plates nearly absent on thorax and head. Coxal depressions not developed. Anterior thoracic spiracles with some associated quadrilocular and simple pores, 3–5 μm in diameter, anterior thoracic spiracles 20 μm in diameter. Hairlike setae, scattered in medial areas of thorax. Quadrilocular and simple pores 3-6 μm in diameter; quadriloculars and simple pores present in wide, long bands within ovisac band and scattered between wax plates elsewhere. Abdominal spiracles five pairs.

DORSUM

Wax plates scattered in single rows. Spines at margin of wax plate elongate 13 μm long. Hairlike setae present in marginal clusters near posterior edges of marginal wax plates, and near anterolateral and anteromedial edge of each dorsomedial wax plate, 19 μm long. Short tubular quadrilocular pores scattered between wax plates, long tubular ducts absent. Anal ring with two rows of pores; longest anal ring seta 36 μm long, anal ring round, 46 μm in diameter.

The species is named after the country of collection of the samples.

COMMENTS

N. brasiliensis is different from other *Newsteadia* species, by having 6-segmented antennae with long, strong, setose hairlike setae on the antennae.

Newsteadia costaricaensis sp. n.

MATERIAL EXAMINED

Holotype: female, Costa Rica, Sierra de la Muerte, El Empalme (2150 m a.s.l.), 1992, Leg: J. Balogh, No. B 62. Paratypes: Five females from the same collection as the holotype, on 5 slides, Nos. B 61, B 62.

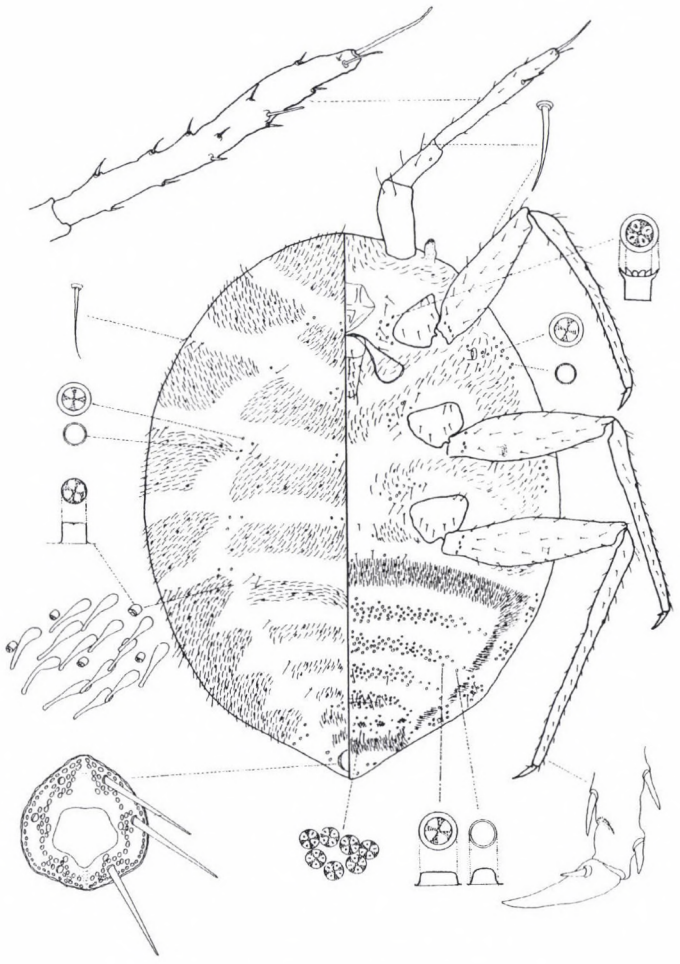


Fig. 4. *N. costaricaensis* sp. n., female

DESCRIPTION OF ADULT FEMALE

Mounted specimen *(Fig. 4):* Holotype adult female 1.2 mm long, 1.0 mm wide. Antennae 3-segemented, 1st segment 176 μm long, 2nd segment 128 μm long, apical segment 336 μm long, basal segments of antennae with strong hairlike setae, apical segment with 15 setose hairlike setae, apical seta 84 μm long, with 34 μm long subapical seta, with a microseta at base, medial sensory seta 35 μm.

VENTER

Labium 208 μm long. Stylet loop as long as labium. Legs: 1st coxa 121 μm long, trochanter-femur 332, tibia-tarsus 457 μm long, claw 38, claw digitules 9 μm long, 2nd

coxa 128 μm long, trochanter-femur 357, tibia-tarsus 473, claw 38, claw digitules 9 μm long, 3rd coxa 144 μm long, trochanter-femur 398, tibia-tarsus 540 μm long, claw 43, claw digitules 10 μm long, with spinelike setae; without flagellate sensory setae near tibial sensorium on all legs; trochanter with 4 sensory pores on each surface. Ovisac band complete; wax plates partly cover thorax and head. Coxal depressions not developed. Base of coxa with hairlike setae, quadrilocular pores and some thick-walled 7 μm long quadrilocular pores. Anterior thoracic spiracles with some quadrilocular and discoidal pores, 3–5 μm in diameter, anterior thoracic spiracles 20 μm in diameter. Quadrilocular and simple pores 4–6 μm in diameter on abdomen; tubular quadrilocular pores short, protruding from derm, associated with wax plates; quadriloculars and simple pores present in wide bands within ovisac band and scattered between wax plates elsewhere. Raw of grouped quadrilocular pores present posterior of vulva, 4 μm in diameter. Abdominal spiracles five pairs.

DORSUM

Wax plates cover most surface. Spines at margin of wax plate elongate 17 μm long. Hairlike setae present in small numbers near posterior edges of marginal wax plates, and near anterolateral and anteromedial edge of each dorsomedial wax plate, 26 μm long. Tubular-like quadrilocular and discoidal pores scattered between wax plates. In wax plates short tubular ducts 4 μm long present, long tubular ducts absent. Anal ring with rows of pores; longest anal ring seta 41 μm long, anal ring 48 μm wide and 49 μm long.

The species is named after the country of collection.

COMMENTS

N. costaricaensis is similar to *N. trisegmentalis,* but differs by having clusters of quadrilocular pores on the venter of the abdomen, by the presence of a subapical seta, and by the absence of long tubular ducts.

Newsteadia floridensis sp. n.

MATERIAL EXAMINED

Holotype: female, USA, Florida, Ocala Nat. For. 26.11.72, Marion Co., Leg: R. D. Kaplan. Paratype: one female from the same collection as the holotype. Types are deposited in the collection of USNM.

DESCRIPTION OF ADULT FEMALE

Mounted specimen *(Fig. 5):* Holotype adult female 1.0 mm long, 0.8 mm wide. Antennae 7-segmented, 1st segment 144 μm long, 2nd segment 102 μm long, apical segment 147 μm long, with clavate setae on the first two segments, with spinelike setae on other segments, apical seta 70 μm long, subapical seta 22 μm long, medial sensory seta 24 μm.

VENTER

Labium 157 μm long. Stylet loop as long as labium. Legs: 1st coxa 96 μm long, trochanter-femur 262, tibia-tarsus 398 μm long, claw 35, claw digitules 8 μm long, 2nd

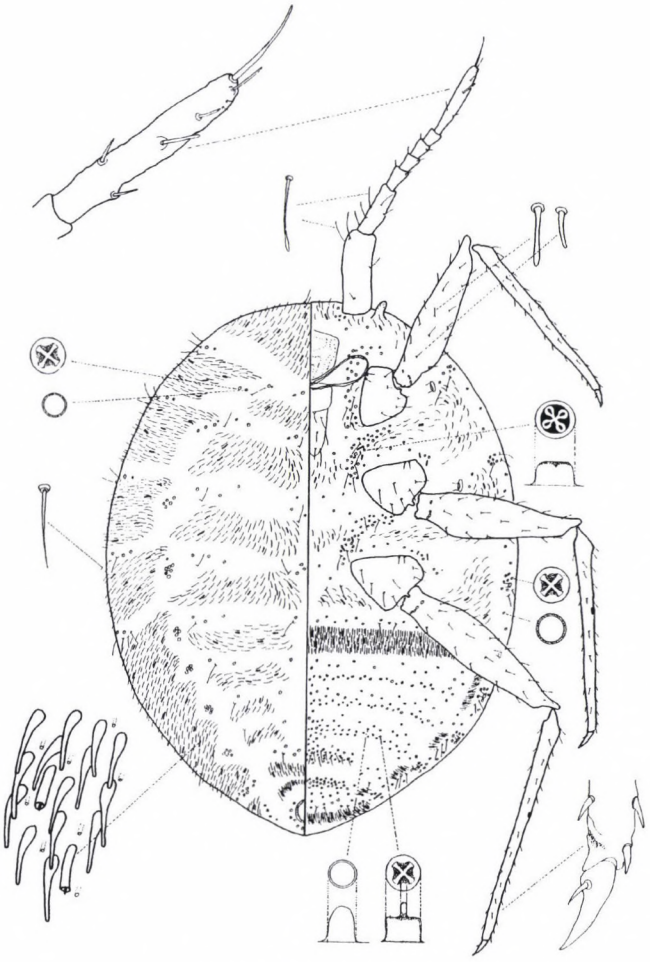


Fig. 5. *N. floridensis* sp. n., female

coxa 103 μm long, trochanter-femur 294, tibia-tarsus 415, claw 36, claw digitules 9 μm long, 3rd coxa 110 μm long, trochanter-femur 336, tibia-tarsus 465 μm long, claw 36, claw digitules 9 μm long, partly with clavate, and with spinelike setae; without flagellate sensory setae near tibial sensorium on all legs; trochanter with 4 sensory pores on each surface. Ovisac band not interrupted in the middle, ovisac wax plate groups on last segments of abdomen present; wax plates in small groups on thorax and head. Coxal depressions with groups of quadrilocular pores, hairlike setae and tubular ducts. Anterior thoracic spiracles with some quadrilocular pores, 6 μm in diameter, anterior thoracic spiracles 23 μm in diameter. Hairlike setae scattered in medial areas of thorax. Quadrilocular and simple pores 3–5 μm in diameter; tubular quadrilocular pores long,

protruding from derm, associated with wax plates club-shaped pores are present, quadrilocular and simple pores present in rows within ovisac band and scattered between wax plates elsewhere. Abdominal spiracles five pairs.

DORSUM

Wax plates interrupted in middle of bands, cover the surface. Spines at margin of wax plate elongate 17 μm long. Hairlike setae present in marginal clusters near posterior edges of marginal wax plates, and near anterolateral and anteromedial edge of each dorsomedial wax plate, 23 μm long. Microtubular ducts scattered, abundant between wax plates, long tubular ducts 14 μm long. Anal ring with two rows of pores; longest anal ring seta 24 μm long, anal ring 48 μm in diameter.

The species is named after the state in the USA where it was collected.

COMMENTS

This specimens were identified as *N. minima* by Morrison, 1952, however *N. floridensis* differs from *N. minima,* by having clavate setae on the first segment of the antenna, and by the presence of groups of quadrilocular pores on the dorsum between the wax plates.

Newsteadia minima Morrison, 1952

MATERIAL EXAMINED

Holotype: female, USA, Durham, N.C. 08. 09. 1944, Leg:. A. S. Pearse, and 3 paratype females from the same collection as the holotype, on three slides.

This species is different from all known species from the Region by widely separated wax plates especially on the abdominal dorsum, and the small number of wax plate seta on the venter of the thorax.

This species was not found in our samples.

COMMENTS

Both species *(N. americana* and *N. minima)* are similar to *N. floccosa* De Geer, 1778, and *N. kanayana* Kawai et Takagi, 1971, from the Palaearctic Region (Kozár, 1998; Kozár and Konczné Benedicty, 1999b). However *N. floccosa* differs from both species by having large group of quadrilocular pores near to coxal bases. *N. kanayana* differs from *N. floccosa, N. americana* and *N. minima* by having wax plates on the dorsum that are, and by having a small number of wax plate setae on the venter of the thorax.

Newsteadia morrisoni sp. n.

MATERIAL EXAMINED

Holotype: female, Costa Rica, Tarbaca (1550 m a.s.l.), 14. 01. 1993, Leg: J. Balogh, No. B 98. Paratypes: Eleven females from the same collection as the holotype on 9 slides, Nos. B 93, B 95, B 96. Seven females, on four slides, Costa Rica, Sierra de la Muerte (1800 m a.s.l.), 24. 01. 1992, Leg: J. Balogh No. B 54, B 55.

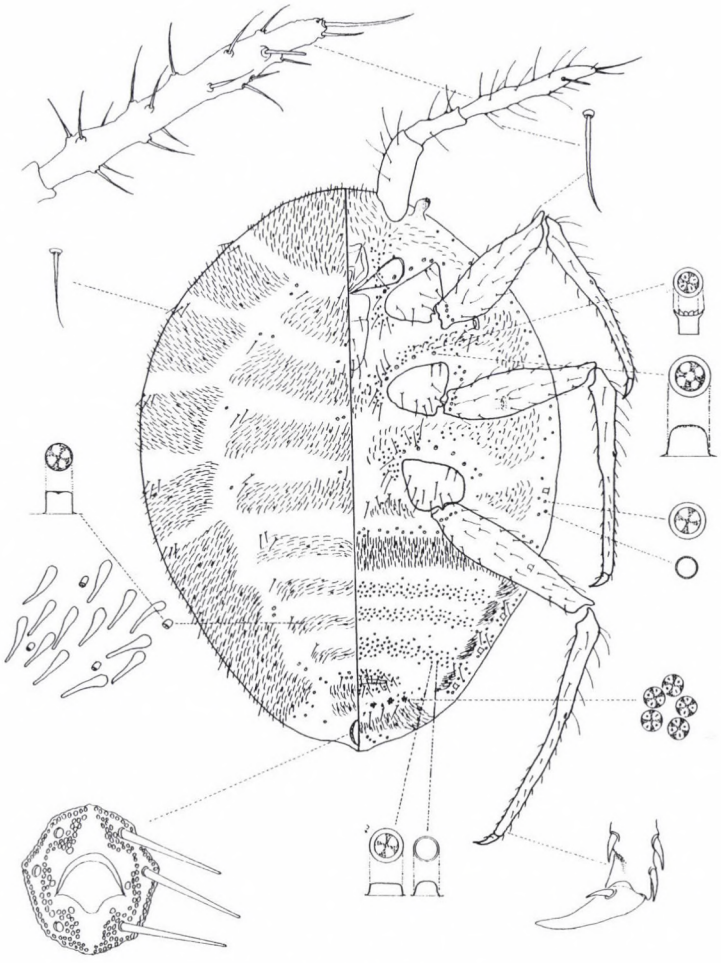


Fig. 6. *N. morrisoni* sp. n., female

DESCRIPTION OF ADULT FEMALE

Mounted specimen *(Fig. 6):* Holotype: adult female 1.3 mm long, 0.9 mm wide. Antennae 3-segemented, 1st segment 186 μm long, 2nd segment 128 μm long, apical segment 314 μm long, with long hairlike setae on all segments, with 19 setae on apical segment, apical seta 99 μm long, subapical seta 49 μm long, with one short subapical seta, medial sensory seta 51 μm.

VENTER

Labium 182 μm long. Stylet loop as long as labium. Legs: 1st coxa 128 μm long, trochanter-femur 332, tibia-tarsus 440 μm long, claw 38, claw digitules 8 μm long, 2nd

coxa 131 μm long, trochanter-femur 349, tibia-tarsus 473, claw 41, claw digitules 8 μm long, 3rd coxa 134 μm long, trochanter-femur 382, tibia-tarsus 538 μm long, claw 38, claw digitules 10 μm long, with hairlike setae on coxae, trochanter-femur, and part of tibia-tarsus, and spinelike setae on other parts, of tibia-tarsus and claw; without flagellate sensory setae near tibial sensorium on all legs; trochanter with 4 sensory pores on each surface. Ovisac band complete; wax plates in groups or bands on thorax and head. Coxal depressions not developed. Anterior thoracic spiracles with some quadrilocular pores, 5 μm in diameter, anterior thoracic spiracles 22 μm in diameter. Hairlike setae, scattered in medial areas of thorax. Quadrilocular pores 6 μm in diameter; tubular quadrilocular pores protruding from derm, associated with wax plates; quadriloculars and simple pores present in wide bands within ovisac band and scattered between wax plates elsewhere. Abdominal spiracles five pairs.

DORSUM

Wax plates cover only part of surface in wide bands. Spines at margin of wax plate elongate 12 μm long. Hairlike setae present in marginal clusters near posterior edges of marginal wax plates, and near anterolateral and anteromedial edge of each dorsomedial wax plate, 22 μm long. Short tubular quadrilocular pores present, and long tubular ducts absent. On the dorsum only simple, and some quadrilocular pores are present. Anal ring with rows of pores; longest anal ring seta 43 μm, anal ring 48 μm wide and 50 μm long.

The species is named after Harold Morrison, the author of the first World monograph of this family.

COMMENTS

N. morrisoni differs from all other species known from the region by the large number of long hairlike setae on all segments of the antennae.

Newsteadia trisegmentalis Howell, 1975

MATERIAL EXAMINED

Holotype: female, USA, Echols Co., Georgia, 07. 03. 1973, on Panicum sp., Leg: S. J. Beshaer, and 2 paratype females from the same collection.

Additional material: There is a female from Florida, Weeki Wachee, Hernando Co. 04. 27. 1975, on *Quercus,* Leg: R. F. Denno, Det. J. A. Davidson and D. R. Miller, No. 2798, and also one female from Mexico, Brownsville, 08. 06. 1987, on orchids and bromeliad debris, Leg: D. Riley, No. 88-06320, all are in USNM collection.

This species (female and larvae) was found in our samples from Venezuela, between Maracay and Caracas, 1973, Leg: J. Balogh, No. Ve 36. There are four larvae in our samples from Antilles, Santa Lucia, 1980, Leg: S. Mahunka, which probably belongs to this species.

COMMENTS

This species is similar to *N. tristani,* the morphological differences will be discussed under the latter species.

Newsteadia tristani (Silvestri, 1924)

MATERIAL EXAMINED

Holotype (on the original slide written "Typus", but according to handwriting it is not sure that it was written by Silvestri) (the original slide contains two specimens, the lectotype designated here, circled by red colour, on the upper left side of the slide) *(Fig 7)* (preadult female): (including one syntype, also a preadult female on the same slide) Costa Rica, San Jose, 01. 1917, remounted by Zs. Konczné Benedicty, 1 preadult female from alcohol, from the same collection (mounted by Zs. Konczné Benedicty), from the collection of Napoli University, Portici, Italy. The species was described on the base of these preadult females (Morrison, 1925). Later Morrison (1952) found an adult female in

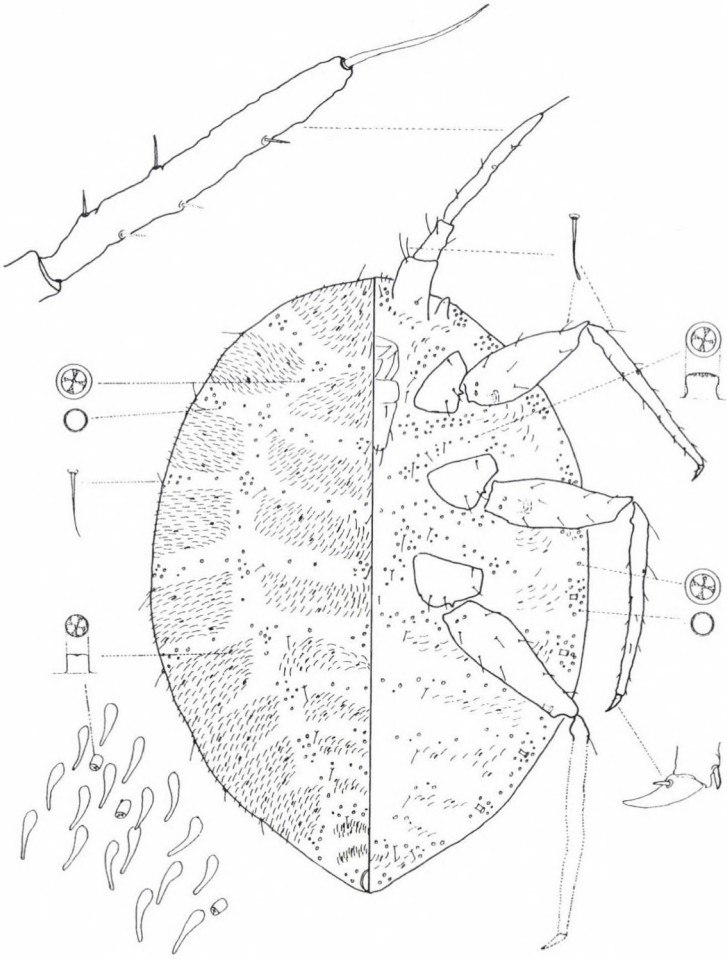


Fig. 7. *N. tristani* (Silvestri, 1924), preadult female

his collection from Mexico Tuxtla Gutierrez, 16. 08. 1948, on orchid, Leg: A. Chapman, No. Laredo 47062, which he "accepted as an example of this species".

Here we present a new, detailed drawing, and description of the type material (preadult female) *(Fig. 7),* drawing, and description *(Fig. 8)* of the female "example of this species" mentioned by Morrison (1952).

Additional material: The species was found from some other places in our samples. One female, from Mexico, Leg: S. Mahunka, 1996, No. 351. Two females (on two slides) and one female with a larva (on one slide), and 4 larvae (on two slides), from Peru, Iquitos, Lacuna, Quistococha, 01. 12. 1971, Leg: J. Balogh, Nos. B5, B6, B7, B17.

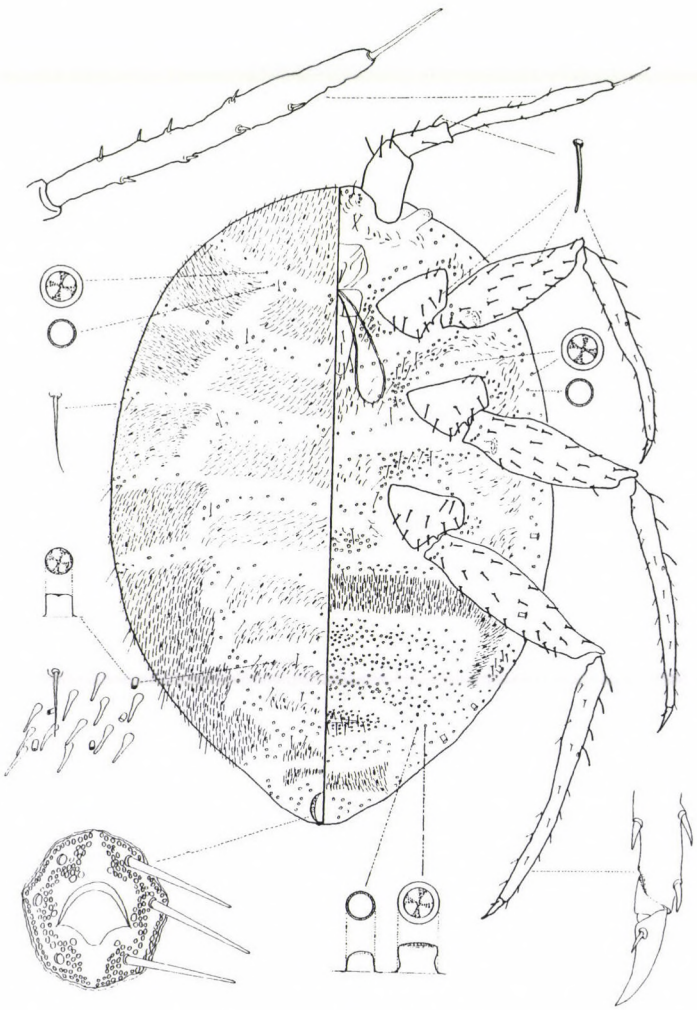


Fig. 8. *N. tristani* (Silvestri, 1924), female

DESCRIPTION OF PREADULT FEMALE

Mounted specimen *(Fig. 7):* Holotype preadult female 1.3 mm long, 0.9 mm wide. Antennae 3-segemented, 1st segment 96 μm long, 2nd segment 66 μm long, and apical segment 214 μm long, with clavate setae on 1st and 2nd segments of the antennae, 30 μm long on 1st and 2nd segments, apical seta 82 μm long with spinelike setae, without subapical, and medial sensory setae.

VENTER

Labium 109 μm long. Legs: 1st coxa 68 μm long, trochanter-femur 208, tibia-tarsus 275 μm long, claw 30, claw digitules 6 μm long, 2nd coxa 72 μm long, trochanter-femur 218, tibia-tarsus 282 μm long, claw 30, claw digitules 6 μm long, 3rd coxa 78 μm long, trochanter-femur 243, other parts broken, with clavate setae on coxa, trochanter-femur and part of tibia-tarsus, and with spinelike setae on other part of tibia-tarsus and claw; without flagellate sensory setae near tibial sensorium; trochanter with 2 sensory pores on each surface. Ovisac band absent; wax plates absent, only some wax plate setae present in groups, and rows. Coxal depressions not developed. Anterior thoracic spiracles with associated cluster of quadrilocular and simple pores, 3–6 μm wide, diameter of anterior thoracic spiracles 22 μm. Hairlike setae few, scattered in medial areas of thorax. Quadrilocular pores 6 μm in diameter; present in groups on thorax, and scattered on abdomen. Five pairs of abdominal spiracles.

DORSUM

Wax plates in wide bands on surface, not divided on midline. Spines at margin of wax plate elongate, with apically rounded apex, 12 μm long. Hairlike setae present near posterior edges of marginal wax plates, and near anterolateral and anteromedial edge of each dorsomedial wax plate. Quadrilocular and simple pores scattered between wax plates, 3–5 μm long, short tubular ducts, 4 μm long, present in wax plates. Anal ring partly destroyed; longest anal ring seta 22 μm long, shorter than length of anal ring; ring 31 μm wide, and 34 μm long.

COMMENTS

The preadult female of *N. tristani* is similar to preadult females of *N. trisegmentalis* (not from type series). The most important difference is the presence of a sensory seta on the apical segment of the *N. trisegmentalis* (which is present in all larval and adult stages). This sensory seta is absent in preadult and adult specimens of *N. tristani.* The setae on the first two segments of the antennae, and coxae of *N. tristani* are long and clavate, but short and not clavate in all stages of *N. trisegmentalis,* as was mentioned by Howell (1975), the latter species also has long tubular ducts.

DESCRIPTION OF ADULT FEMALE

Mounted specimen *(Fig. 8):* Female (Morrison's "example of this species") 1.3 mm long, 0.9 mm wide. Antennae 3-segmented, 1st segment 128 μm long, 2nd segment

102 μm long, and apical segment 352 μm long, with 3 clavate setae on 1st and 2nd segments, 18–23 μm long, with 7 spinelike setae 12 μm long on apical segment, apical seta 82 μm long, without subapical, and medial sensory seta.

VENTER

Labium 154 μm long. Stylet loop as long as labium. Legs: 1st coxa 102 μm long, trochanter-femur 340, tibia-tarsus 423 μm long, claw 43, claw digitules 8 μm long, 2nd coxa 122 μm long, trochanter-femur 365, tibia-tarsus 457 μm long, claw 46, claw digitules 8 μm long, 3rd coxa 134 μm long, trochanter-femur 415, tibia-tarsus 539 μm long, claw 48, claw digitules 13 μm long, legs with clavate setae on coxae, trochanter-femur and partly on tibia-tarsus, on other part of tibia-tarsus with spinelike setae; without flagellate sensory setae near tibial sensorium; trochanter with 4 sensory pores on each surface. Ovisac band not interrupted, but short, ovisac wax plate groups on last segments of abdomen absent; wax plates very few on the thorax and head. Coxal depressions weakly developed, mesad of each coxa, with a cluster of hairlike setae, wax spines, and many quadrilocular pores. Anterior thoracic spiracles without quadrilocular and simple pores, diameter of anterior thoracic spiracles 35 μm. Hairlike setae few, scattered in medial areas of thorax. Quadrilocular and discoidal pores 4–6 μm in diameter; quadrilocular and discoidal pores present in wide bands within ovisac band and scattered between wax plates. With five pairs of abdominal spiracles.

DORSUM

Wax plates in wide bands, partly divided in midline. Spines at margin of wax plate elongate, with apically rounded apex, 16 μm long. Hairlike setae present in marginal clusters near posterior edges of marginal wax plates, and near anterolateral and anteromedial edge of each dorsomedial wax plate, 24 μm long. Quadrilocular and simple pores scattered between wax plates, 3–5 μm in diameter, tubular ducts short, 4 μm long, present in wax plates. Anal ring with rows of pores; longest anal ring seta 38 μm long, shorter than length of anal ring; ring 54 μm wide, and 60 μm long.

COMMENTS

N. tristani (although described only on the basis of preadult females), is a distinct species, distributed in different parts of Central and South America. The new larvae found are nonspecific with the larvae from type series, and the females found altogether with larvae makes possibly to identify this species and accept as valid.

Newsteadia tropicalis sp. n.

MATERIAL EXAMINED

Holotype: female (marked with red circle, on right side of the slide) (including one paratype female on the same slide, Brazil, Leg: J. Balogh, 1995, No. B 76. Paratypes: two females from the same collection as the holotype, on two slides, Nos. B 2, B 76.

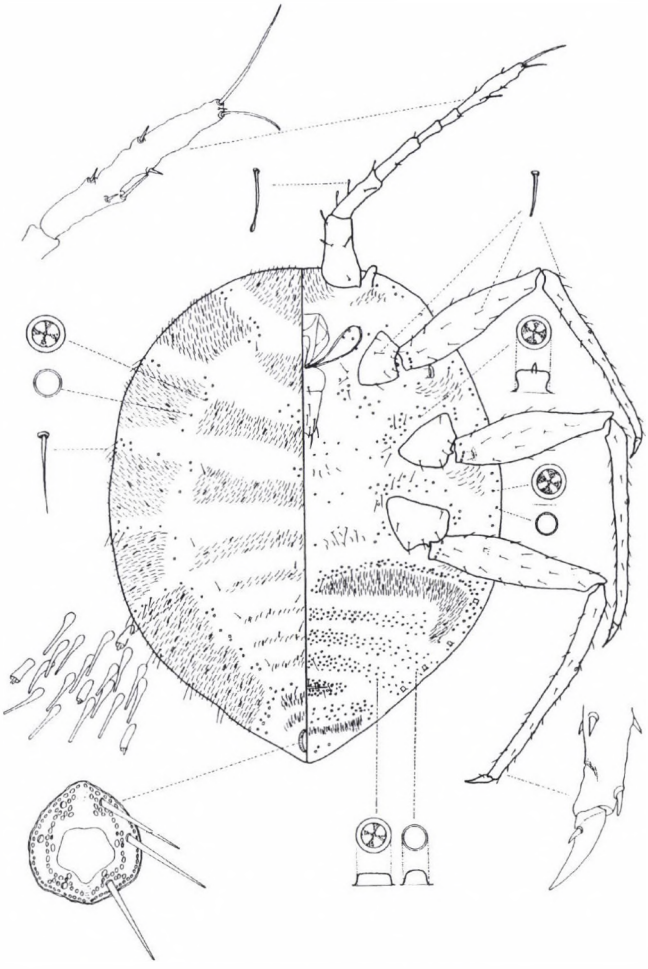


Fig. 9. *N. tropicalis* sp. n., female

DESCRIPTION OF ADULT FEMALE

Mounted specimen *(Fig. 9):* Holotype adult female 1.1 mm long, 0.9 mm wide. Antennae 6-segmented, 1st segment 176 μm long, 2nd segment 118 μm long, apical segment 182 μm long, with clavate setae on first two segments, with 7 spinelike setae on next segments, apical seta 96 μm long, subapical seta 38 μm long, medial sensory seta 33 μm.

VENTER

Labium 160 μm long. Stylet loop as long as labium. Legs: 1st coxa 102 μm long, trochanter-femur 349, tibia-tarsus 432 μm long, claw 38, claw digitules 6 μm long, 2nd coxa 115 μm long, trochanter-femur 374, tibia-tarsus 467, claw 38, claw digitules 6 μm long, 3rd coxa 122 μm long, trochanter-femur 398, tibia-tarsus 481 μm long, claw 41, claw

digitules 7 μm long, partly with clavate, and partly with spinelike setae; without flagellate sensory setae near tibial sensorium on all legs; trochanter with 4 sensory pores on each surface. Ovisac band interrupted in middle, short, ovisac wax plate groups on last segments of abdomen absent; wax plates in small groups on thorax and head. Coxal depressions not developed. Anterior thoracic spiracles with some associated quadrilocular pores, 4 μm in diameter, anterior thoracic spiracles 22 μm in diameter. Hairlike setae scattered in medial areas of thorax. Quadrilocular and simple pores 3–5 μm in diameter; tubular quadrilocular pores long, protruding from derm, associated with wax plates; quadriloculars and simple pores present in wide, bands within ovisac band and scattered between wax plates elsewhere. Abdominal spiracles five pairs.

DORSUM

Wax plates forming bands on surface. Spines at margin of wax plate elongate, 16 μm long. Hairlike setae present in marginal clusters near posterior edges of marginal wax plates, and near anterolateral and anteromedial edge of each dorsomedial wax plate, 24 μm long. Tubular quadrilocular pores scattered between wax plates, long tubular ducts 16 μm long. Anal ring with two rows of pores; longest anal ring seta 36 μm long, anal ring round 48 μm in diameter.

The species is named after the tropical region where it was collected.

COMMENTS

N. tropicalis is similar to *N. brasiliensis,* but differs by having clavate setae on the first segments of the antennae, and by presence of long tubular ducts.

Key to adult females of *Newsteadia* found in these regions

1. Antennae three segmented 2
   – Antennae more than three segments 6
2. Antennal setae clavate 3
   – All antennal setae apically acute 4
3. All antennal setae long, clavate *borhidii* sp. n.
   – Antennal seta clavate only on the first and second segment *tristani* (Silvestri, 1924)
4. All antennal setae long, hairlike *morrisoni* sp. n.
   – Some antennal seta spinelike 5
5. With long tubular ducts, without clusters of quadrilocular pores, subapical seta absent *trisegmentalis* Howell, 1975
   – With short tubular ducts, with clusters of quadrilocular pores, subapical seta present *costaricaensis* sp. n.
6. Ovisac band interrupted 9
   – Ovisac band not interrupted 7
7. Tubular ducts short *floridensis* sp. n.
   – Tubular ducts long 8

8. Wax plates densely cover of the venter the thorax, wax plate bands on dorsum not interrupted *americana* Morrison, 1925
   – Wax plates not cover of the venter the thorax, wax plate bands on dorsum interrupted *minima* Morrison, 1952
9 Part of setae on antennae clavate, with long tubular ducts *tropicalis* sp. n.
   – Setae on the antenna strong, setose hairlike, with short tubular ducts *brasiliensis* sp. n.

Zoogeographic Considerations

With the data presented here, and considering earlier reports, the species number of *Newsteadia* in the Nearctic and Neotropic Regions has increased to 10. Two species *(N. americana, N. floridensis,* and *N. minima)* are distributed only in the Nearctic Region,

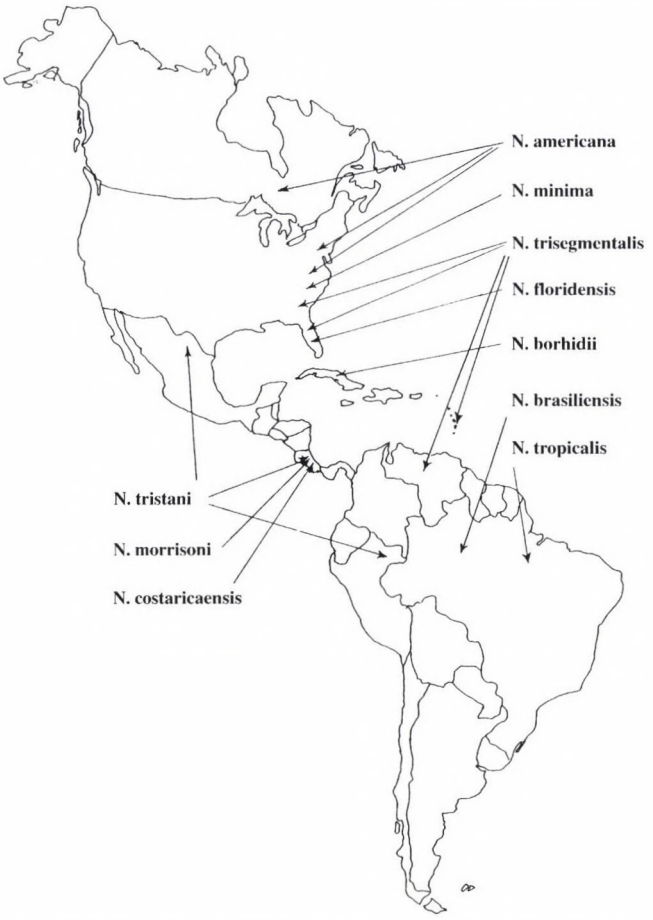


Fig. 10. Distribution of the *Newsteadia* species in the Nearctic and Neotropic Regions

six others *(N. costaricaensis, N. morrisoni, N. borhidii, N. brasiliensis, N. tristani* and *N. tropicalis)* are only in the Neotropic Region and one species is *(N. trisegmentalis)* in both regions.

In the Nearctic and Neotropic Regions most of the species (6) were found in Central America and the northern parts of South America *(Fig. 10). N. trisegmentalis* has the widest distribution, and presents a link between North, Central and South America on eastern side of the continent through the Caribbean Islands. *N. tristani* presents a link between North, Central and South America on the western side of the continent. *N. americana* is distributed widely in the north-eastern part of North America.

With the new species added to the World list, the total number of the extant species has increased to 37, with two fossil species making the number 39. A worldwide zoogeographical analysis will be undertaken in the future, when treatments of *Newsteadia* from Australian and Pacific, Oriental and Palaearctic Regions (Kozár and Konczné Benedicty, 1999a, b), and Ethiopian Region (Miller and Kozár, 1999, in preparation) are published.

Acknowledgements

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Acta Phytopathologica et Entomologica Hungarica 36 (1–2), pp. 143–153 (2001)

Influence of Mint and Peppermint on *Tetranychus urticae* and Some Predacious Mites of the Family *Phytoseiidae* (Acari: Tetranychidae: Phytoseiidae)

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The deterrent and toxicity effects of mint, *Mentha virdis* L. and peppermint, *Mentha piperita* L. on *Tetranychus urticae* Koch were studied under laboratory conditions. *M. virdis* was more potent for *T. urticae* than *M. piperita,* with a significant increase in repellency. Leaf discs treated with increasing concentrations of both materials showed reduction in the total numbers of eggs laid. A high percentage of *T. urticae* mortality was recorded in case of *M. virdis*. The direct toxicity of both essential oils to the female of the predacious mites namely *Typhlodromus athiase* Porath and Swirski *Phytoseius finitimus* Ribaga, *Amblyseius barkeri* (Hughes), *Amblyseius zaheri* Yousef and El-Borolossy, *Amblyseius yousefi* Zaher and El-Borolossy and *Amblyseius deleoni* (Muma and Denmark) were tested. At LC_{50} level, *M. virdis* was the most toxic to females *A. yousefi* and the least to females *T. athiasae.* With the exception of *A. zaheri, M. piperita* proved to be more toxic to the predacious mites tested than *M. virdis.* The results obtained chemically and biologically, may suggest that the higher percentage of the hydrocarbons of *M. virdis* were responsible for the toxic effect.

Keywords: *Mentha piperita, Mentha virdis,* Phytoseiidae Predacious mites, *Tetranychus urticae.*

Aromatic plants have traditionally been used in folk medicine. Recently, the interest of the biological activities of extracts has been the subject of intense scientific investigation. Essential oils drived from many plants are known to possess biological activity against prokaryotic (Deans and Ritchie, 1987) and eukaryotic organisms (Konstantopoulou et al., 1992). The existence of different chemotypes, based on qualitative differences within a taxon, is a common feature in most *Mentha* species and hybrids (Kokkini, 1991). Summarizing research published to date, it can be concluded that the main monoterpenes which characterize the essential oil composition of the different species and hybrids are either cyclic C-2 (such as carvone, hydrocarvone) or C-3 (such as menthone, pulegone) substituted compounds (Kokkini, 1992).

Recently, mites infesting food products became a dangerous problem, also the rise of resistance among its population implied the necessity need to look for combinations of acaricides of different modes of action as alternative methods of mite control, to face the serious problems created by using acaricides. The utilization of some plants against the mite attack, have prompted us to initiate a program for using plant extracts for the biological activity. Among these botanicals, the essential oils which exhibit antifeedant activity and toxic to some important insects (Su et al., 1972; Don-Pedro, 1985; Tare and Sharma, 1988). The effect of the essential oils on pest mites has been studied by Amer et al. (1993); Ibrahim et al. (1993); Perrucci (1995); El-Gengaihi et al.

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(1996); Amer et al. (in prep.). The role of phytoseiid mites as predators of phytophagous mites and insects on various kinds of crops is well documented (Huffaker et al., 1970). However, the phytoseiid predators, *A. barkeri, A. zaheri, T. athiasae, A. deleoni, A. yousefi* and *P. finitimus* have been evaluated for control of spider mites on crops and orchards with promising results (Swirski et al., 1967; El-Banhawy, 1974; Momen, 1995; Abou-Elella, 1998; Momen and El-Borolossy, 1999). Therefore, in the presence of essential oil applications, biological control of spider mites may be achieved by the selective use of essential oil that are less toxic to natural enemies than to pest species. The present work was carried out to complete previous work discussed the activity of some essential oils on the pest *T. urticae* which is very harmful to agriculture. Knowledge of the effects of both materials tested on natural enemies of phytophagous mites and insects is also provided.

Materials and Methods

Plant materials

The essential oil of mint and peppermint plants was obtained during the last week of June 1997 from plants grown in the Experimental farm of National Research Centre at Giza.

Preparation of the oil

The air-dried plant material (aerial parts) was pulverized and the essential oils isolated after hydrodistillation for 3 hours using Clevenger apparatus. The freshly distilled oil of each plant was dehydrated over anhydrus sodium sulphate and then subjected to GC-MS analysis under the following conditions: Apparatus: Varion 3400 gas chromatograph with mass selective detector operated in electron ionization mode at 70 ev. The column used for oil separation was a fused silica DB-5 column, 30 m x 0.25 mm i-d., film thickness 0.25.

Chromatographic investigation of the volatile oil. Mass spectra were obtained by a Hewlett-Packard 5890 gas chromatograph coupled with mass detector. Carrier gas: helium at 1 ml/min., temp. programming 60–200 °C at rate of 3 °C/min.; chart speed: 0.6 cm/min., injection part temp.: 150 °C; split ratio 100:1; start stop masses 39–250; electron multiplier voltage 1800 ev. Identification of the constituents was performed by comparison of their retention times and mass spectral fragmentations with those of the published data by Adams (1989). Quantitative determination was carried out based on peak area measurements.

Preparation of the emulsions

Emulsions of mint *(Mentha virdis)* and peppermint *(Mentha piperita)* were prepared by mixing of Triton-x 100. Different concentrations of the product were prepared and tested against the adult females of mites.

Maintenance of mite stock cultures

The stock cultures of *T. urticae* were collected from infested lima bean (*Phaseolus vulgaris* L.) in the laboratory at N. R. C. Cairo. The following predacious mites were used in our studies, *A. zaheri* and *A. barkeri* were found on leaves of eggplants; *P. finitimus* was collected from fig oechards; *A. deleoni* and *T. athiasae* were found on mango leaves; *A. yousefi* was found on leaves of *Zizyptus spina-christi,* L. All these strains have been maintained in the laboratory for 3 years. All strains were kept separately on detached bean leaves *P. vulgaris* placed on wet cotton wool with *T. urticae* as prey.

Treatment

REPELLENCY AND TOXICITY TEST PROCEDURE FOR ADULT FEMALES OF *T. URTICAE*

Raspberry leaf discs (3 cm in dia.) were placed with the lower surface upwards in a petri-dish lined with moist cotton wool. One half of each disc was treated separately with selected concentrations of both oils, while the other served as a control. Ten adult females of *T. urticae* were then introduced into the middle of each leaf disc. Ten replicates leaf discs were used per concentration. Orientation of the females *T. urticae* on treated and control discs recorded after 24, 48 and 72 h after treatment. The number of eggs laid on both sides and the percentage mortality of adult females were recorded after 72 h. The repellency was calculated according to Lwand et al. (1985).

TOXICITY AND BIOLOGICAL EFFECTS OF MINT AND PEPPERMINT ON ADULT FEMALES OF *T. URTICAE*

Newly emerged females *T. urticae* were transferred singly on raspberry leaf discs treated with different concentrations of mint and peppermint. The total number of eggs laid were recorded over a period of 10 days. The mortality of the females was also recorded. Each concentration was replicated 20 times and a similar number of discs treated with water only as a control. The different indices were calculated as reported by Lundgren (1975).

DIRECT EFFECT ON SOME PREDACIOUS MITES

Adult females of 6 predators species *(A. barkeri, A. deleoni, A. zaheri, A. yousefi, T. athiasae* and *P. finitimus)* were sprayed with different concentrations from both materials using glass atomizer. Females were confined on the lower surfaces of detached raspberry clean leaves (5 cm in dia.) while the upper surfaces were placed on cotton saturated with water. Each test contained 5 concentrations and each concentration had 4 replicates (20 females/replicate). In every test, a control was included. Mortality was recorded 48 h after application. Corrected mortality counts according to Abbott's formula (1925) and were statistically analysed by Finney (1952).

All the experiments reported herein were carried out the laboratory at 27±2 °C and 70–75% R. H.

Results and Discussion

Chemical constituents of mint and peppermint oils

Qualitative and quantitative analysis of mint *(M. virdis)* and peppermint *(M. piperita)* essential oils showed that both oils were mainly characterized by high concentration of total terpene compounds (88.53 and 92.67%) respectively, and low concentration of sesquiterpenes (11.48 and 7.34%) respectively, although their individual levels varied, in particular the concentration of some components such as, menthon (1.999 and 25.145%) respectively, in addition, the variation in the individuals inside either oils.

Tables 1, 2, 3, 4 and *Figs 1, 2* also show that mint oil was mainly characterized by high concentration of carvone (57.351%), while menthone and menthol represented the main components in peppermint oil since they formed 25.145 and 21.633% of oil content respectively.

Repellency, mortality and oviposition deterrence

Table 5 shows that mint oil at all concentrations used, strongly deterred *T. urticae* adult females. At the lower concentration of mint oil, a considerable percent of *T. urticae* was recorded on treated half. Mortality was extremely high at 2% conc. after 72 h and was slightly at 0.125% conc. Percent repellency gradually decreased with peppermint oil concentrations. Mortality was nil and the number of eggs laid by females *T. urticae* after 72 h of treatment varied according to concentrations of peppermint oil *(Table 5)*. No eggs were laid in case of mint oil. Harwood et al. (1990) demonstrated that reduced growth in larvae of the noctuid *Peridroma saucia* was the result of feeding inhibition in larvae fed menthone and pulegone and moulting abnormalities in larvae receiving menthol, as well as completely inhibited pupation. The essential oil of *M. piperita* was active against adult of *Tribolium castaneum* (Hbst) and toxic also to the newly hatched larvae of *Pectinophora gossypiella* (Saund.) and *Earias insulana* (Boisd.) (Shaaya et al., 1991; Hewady et al., 1994). Similar result has been also reported, that the essential oil of *M. piperita* and some of its main constituents (menthone and menthol) exhibited powerful acaricidal activities against *Tyrophagous longior* by direct contact and by inhalation (Perrucci et al., 1996). *Tables 1, 2* show that the hydrocarbon terpenes and hydrocarbon sesquiterpenes content in mint was higher than on peppermint oil.

Chavan (1984) and Siddiqui et al. (1988) demonstrated that the hydrocarbon fraction isolated from dried neem leaves and twigs possessed larvicidal activity against mosquitoes. Similar results were recorded on *T. urticae* by El-Gengaihi et al. (1999), showing that the leaves of Moghat contained some hydrocarbons, so these hydrocarbons may be responsible for the effect itself or its occurrence with other hydrocarbons may cause synergistic effect, and finally increased activity.

Concentration effects on reproduction and mortality

Reproduction of *T. urticae* was greatly affected by both oils treatment. Significant reduction in the total number of eggs laid during 10 days period were found for all

Table 1

Hydrocarbon terpenes content in mint and peppermint essential oils

Peak No.	Mint oil	%	Peak No.	Peppermint oil	%
1	α-Thujene	0.011	1	Tricyclene	0.047
2	α-Pinene	0.421	2	α-Pinene	2.576
3	Camphene	0.110	3	Camphene	0.078
4	Sabinene	0.230	4	Sabinene	0.752
5	β-Pinene	0.547	5	β-Pinene	2.185
6	Myrcene	0.240	6	Myrecene	0.510
8	D-Limonene	14.162	8	D-Limonene	3.538
9	cis-Ocimene	0.028	10	cis-Ocimene	0.065
10	trans-Ocimene	0.005	11	trans-Ocimene	traces
11	γ-Terpinene	0.006	12	γ-Terpinene	0.035
	Unknown	0.080		Unknown	0.905
	Total	15.840		Total	10.746

Table 2

Oxygenated terpenes content in mint and peppermint oils

Peak No.	Mint oil	%	Peak No.	Peppermint oil	%
7	2-Octanol	0.369	7	3-Octanol	1.159
12	cis-p-menth-2-en-1-01	0.021	9	Cineole	0.123
13	trans-Linalool oxide	0.014	13	Cis-p-menth-2-en-1-01	0.055
14	α-Pinene oxide	0.074	14	Cis-Linalool oxide	0.008
15	Linalool	0.076	15	Fenchone	0.044
16	Menthone	1.997	16	Linalool	0.009
17	trans-Dihydro terpineol	1.591	17	Camphor	0.122
18	Neomenthol	0.575	18	Menthone	25.145
19	Dihydro carveol	0.138	19	Isomenthone	8.379
20	new iso-Dihydro carveol	0.036	20	Newmenthol	8.782
21	Carvone	57.351	21	Menthol	21.633
22	Isomenthyl acetate	0.641	22	α-Terpineol	0.204
23	Terpinenyl acetate	6.030	23	Pulegone	3.252
24	cis-Carvyl acetate	0.877	24	Pipritone	3.213
27	cis-Jasmone	0.192	25	Bornyl acetate	3.707
29	Ionone	0.220	26	Isomenthyl acetate	0.373
	Unknow	2.481	27	trans-Carvyl acetate	0.056
	Total	72.683	29	Eugenol	traces
			30	cis-Carvyl acetate	0.114
			33	Jasmone	0.082
				Unknown	5.514
				Total	81.919

Table 3

Hydrocarbon sesquiterpenes content in mint and peppermint oils

Peak No.	Mint oil	%	Peak No.	Peppermint oil	%
25	β-Bourbonene	3.065	28	D-Elmene	0.190
26	β-Cubebene	0.907	31	Copaene	0.088
28	β-Caryophyllene	2.704	32	β-Bourbonene+β-cubebene	1.129
30	β-Gurjunene	0.253	34	β-Caryophyllene	2.068
31	Aromadendrene	0.545	35	α-trans-Bergamotene	0.159
32	β-Farnesene	0.147	36	Aromadendrene	0.033
33	γ-Muurolene	0.199	37	β-Farnasene	0.156
34	D-Germacrene	0.163	38	Germecrene D	1.050
35	α-Muurolene	0.125	39	α-Longipinene	0.081
36	α-Farnesene	0.012	40	β-Bisabolene	traces
37	γ-Cadinene	0.112	41	γ-Cadinene	0.332
38	15-cis-Calamenene	0.076		Unknown	0.518
	Unknown	1.444		Total	5.824
	Total	9.752			

Table 4

Oxygenated sesquiterpenes content in mint and peppermint oils

Peak No.	Mint oil	%	Peak No.	Peppermint oil	%
39	Caryophyllene oxide	1.160	42	Nerolidol	0.047
40	τ-Cadinol	0.025	43	Spathulenol	0.013
41	α-Cadinol	0.033	44	Caryophyllene oxide	0.384
	Unknown	0.507	45	τ-Cadinol	0.066
	Total	1.725	46	α-Cadinol	0.058
			47	α-Bisabolol	0.019
				Unknown	0.934
				Total	1.521

the concentrations tested *(Table 6)*. The depression in total number of eggs with high concentrations could be attributed to feeding inhibition and irritant effects of the formulation, causing depression on reproduction activity. Similar results have been reported by Gulati and Mathur (1995) indicating that mentha leaf powder was effective in bring about a decrease in the fecundity of *Tyrophagous putrescentiae* (Schrank) and reducing the mean egg numbers to 25.49/female as compared to 98.16 egg/female in the control. Mortality percentage reached 100% with mint oil, however, with low concentration, the effect was pronounced with mint oil than peppermint oil. Mansour et al. (1986) reported that the most effective oil on *Tetranychus cinnabarinus* (Boisd) was *M. piperita.* In contrast the oil was not effective on *Acanthoscelides obtectus* Say a pest of bean *Phaseolus vulgaris* (L.) (Regnault-Roger and Hamraoui, 1993). The same authors in (1995)

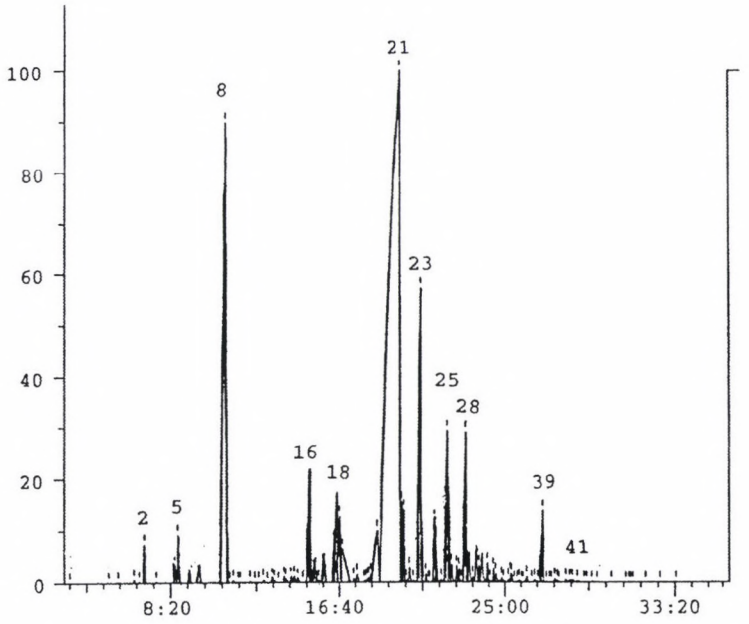


Fig. 1. Chromatogram of mint essential oil

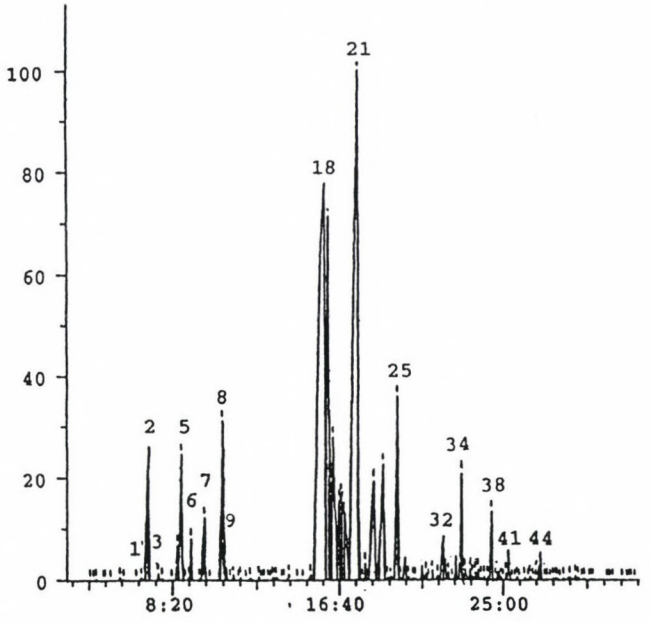


Fig. 2. Chromatogram of peppermint essential oil

suggested that lipid as well as nonlipid allelochemical, such as phenolics, or nonprotein amino acids or flavonoids, may be involved in the toxicity of hydrodistillated and intact plant extracts from *M. piperita* to *A. obtectus.*

Table 5

Phagodeterrent activity of mint and peppermint oils against females of *T. urticae*

% Concentrations	% Distribution of mites on treated leaf part after			% Mortality after	Avg. No. of eggs/female after		% Repellency
	24 h	48 h	72 h	72 h	72 h		
					T	C	
				Mint oil			
2	0	0	0	75	0	0.05	100
1	0	0	0	40	0	0.15	100
0.5	0	0	5	20	0	0.65	100
0.25	0	0	15	15	0	0.7	100
0.125	0	10	20	10	0	1.55	100
				Peppermint oil			
2	15	15	20	0	0.05	0.5	90
1	20	20	20	0	0.2	1.4	85.7
0.5	25	30	35	0	0.3	1.4	78.6
0.25	35	45	50	0	0.85	1.95	56.4
0.125	45	55	55	0	1.05	1.95	46.2

C = control; T = treated

Table 6

Effect of mint and peppermint oils on reproduction and mortality of *T. urticae*

% Concentrations	Mint oil			Peppermint oil		
	Total no. of eggs/female/10 days ± S. E.	% ODI	% Mortality after 10 days	Total no. of eggs/female/10 days ± S. E.	% ODI	% Mortality after 10 days
2	0±0	100	100	0±0	100	58.33
1	0.55±0.11	98.14	80	0±0	100	21.43
0.5	5.7 ±0.33	82.24	66.67	3.90±0.22	87.5	18.18
0.25	6.2 ±0.33	80.83	46.67	7.65±0.25	76.87	0.00
0.125	8.75±0.25	73.98	26.67	9.0 ±0.15	73.33	0.00
Control	58.5±0.45	–	–	85.5±0.45	–	0.00

High signigicant $\leq 0.01\%$

Direct effect of mint and peppermint oils on adult females of some predacious mites

The data obtained in *Tables 7* and *8* show that adult females of *A. yousefi* was more sensitive ($LC_{50} = 2.954\%$) to mint oil, while females of *T. athiase* was less susceptible ($LC_{50} = 16.15\%$). Results indicated also that peppermint oil was more toxic to the predacious mites tested than mint oil except for females of *A. zaheri.* Sopp et al. (1990)

Table 7

Toxicity of mint oil to females of some predacious phytoseiid mites

Species	% LC_{50}	% LC_{90}	Slope	Toxicity index at		No. of folds compared with *T. athiasae* at	
				LC_{50}	LC_{90}	LC_{50}	LC_{90}
A. yoseffi	2.954	11.81	2.13	100	100	5.47	15.66
A. zaheri	5.530	42.50	1.45	53.42	27.79	2.92	4.35
P. finitimes	6.882	14.77	3.86	42.92	79.96	2.35	12.52
A. barkeri	8.796	31.25	2.33	33.58	37.79	1.84	5.92
A. deleoni	12.09	114.2	1.31	24.43	10.34	1.34	1.62
T. athiasae	16.15	184.9	1.21	18.24	6.39	1.00	1.00

Table 8

Toxicity of peppermint oil to females of some predacious phytoseiid mites

Species	% LC_{50}	% LC_{90}	Slope	Toxicity index at		No. of folds compared with *A. zaheri* at	
				LC_{50}	LC_{90}	LC_{50}	LC_{90}
T. athiasae	1.90	65.72	0.83	100	8.23	13.3	11.22
A. yoseffi	2.314	6.277	2.95	82.11	86.19	10.92	117.48
P. finitimes	2.890	9.504	2.47	65.74	56.92	8.74	77.59
A. deleoni	2.92	19.14	1.57	65.07	28.27	8.65	38.53
A. barkeri	3.72	5.41	7.89	51.08	100	6.79	136.30
A. zaheri	25.27	737.40	0.87	7.52	0.73	1.00	1.00

reported that *Phytoseiulus persimiles* A. H. was relatively unaffected by the beta-acids showing only a small reduction in the number of eggs laid. Mansour et al. (1993) revealed that RD9-Repelin was highly toxic to *T. athiasae* but Margosan-O and Azatin were not toxic. Research carried out by Momen and Amer (1994) and Momen et al. (1997) demonstrated that Lupin and canna extracts were toxic to females *A. barkeri,* also Neem Azal-F appeared to be harmless for *A. barkeri* and *A. zaheri.* Pyrethrum and rotenone had a negative effect on *Typhlodromus exhilaratus* Ragusa, and also stinging nettle and bitter wood were not effective on the predator (Tsolakis et al., 1997).

Conclusion

On scrutinizing our results, it will be shown that *M. virdis* is satisfactory as regards both high mortality and low reduction of fecundity for *T. urticae. M. virdis* was more toxic to *T. urticae* than to phytoseiid predators studied. In contrast, *M. piperita* was

more effective on most phytoseiid predators than *M. virdis,* as well as it was considered to be less toxic to *T. urticae* than *M. virdis.* However, *M. virdis* can protect agricultural crops by direct or delayed pesticidal effect, through increased adult mortality and inhibition of reproduction. In integrated control programmes, therefore a careful choice of essential oil should be made to harm phytoseiid populations as little as possible. Consequently, knowledge about possible adverse affects of specific essential oil is essential. Field and laboratory data on the toxicity of such compounds to predacious mites should be obtained.

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Repellent and Oviposition-Deterring Activity of Rosemary and Sweet Marjoram on the Spider Mites *Tetranychus urticae* and *Eutetranychus orientalis* (Acari: Tetranychidae)

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The deterrent and toxic effect of two essential oils, *Majorana hortensis*. Moench and *Rosmarinus officinalis* L. on the two tetranychid mites *Tetranychus urticae* Koch and *Eutetranychus orientalis* (Klein) were studied under laboratory conditions. Both materials used were more potent for *E. orientalis* than against *T. urticae* with a significant increase in repellency.

Leaf discs treated with increasing concentrations of the two oils showed increased mortality of both spider mites and reduction in the total numbers of eggs laid. This result could be due to the oil of the higher oxygenated compounds content that was more effective in this respect.

Keywords: *Tetranychus urticae, Eutetranychus orientalis, Majorana hortensis, Rosmarinus officinalis*, essential oil.

The use of chemical pesticides offers problems such as atmospheric pollution and residual toxicity in food. For these reasons, research on non-contaminating pesticides is needed. Research in recent years has been turning more towards selective biorational pesticides, such as plant-derived compounds, because they are perceived to be safer than the synthetics (Arnason et al., 1989). Few studies have been done on such plants having acaricidal influences are assumed to be acceptable because they are alterable in nature (Schauer and Schmutterer, 1981; Amer 1984; Rasmy 1985; Amer et al., 1989; Dimetry et al., 1990). Among these botanicals, the essential oils exhibit antifeedant activity and toxic to some important insects (Sy et al., 1972; Purohit et al., 1983; Don-Pedro, 1985; Tare and Sharma, 1988).

In Egypt, very few research has been reported on the effect of the essential oils on mites (Amer et al., 1993; Ibrahim et al., 1993; El-Gengaihi et al., 1996).

Both *Majorana hortensis* Moench and *Rosmarinus officinalis* L. (Fam. Labiatae) are considered as two of the most valuable aromatic and medicinal plants. Rosemary oil has been reported by Guenther (1961) as an insect repellent, and as an ingredient in rubefacient liniments. He stated also that its oil contains: α-pinene, camphene, cineole, dipentene, camphor, borneol and sesquiterpene compounds. Sweet marjoram is used medicinally as carminative and stimulant due to its strong, highly aromatic and pleasing odor (Guenther, 1961). Marjoram oil contains terpinene 4-01, α-terpinene, D-limonene, linalool, linalyl acetate, P-cymene, β-pinene and α-terpineol.

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This is the first report, dealing with the acaricidal properties and biological activity of both *M. hortensis* and *R. officinalis* on two of the most serious pests in Egypt, namely *Tetranychus urticae* Koch and *Eutetranychus orientalis* (Klein) under laboratory condition.

Materials and Methods

Plant materials

Both plants essential oils (rosemary and sweet marjoram) were obtained in June and November 1998, respectively from the air-dried herbs about seven to nine months old, originally grown as plants in the Experimental Farm of National Research Center at Giza.

Preparation of the oil

The essential oil was extracted by submitting the air-dried herb of each to stem distillation for 3 hours in a Clevenger apparatus. The oil collected from each was dehydrated over anhydrous sodium sulfate and subjected to GC-MS analysis.

Chromatographic investigation of the volatile oil

The obtained essential oils were subjected for GC-MS analysis under the following conditions:

GC-MS Finnigan mat SSO 7000 Digital DEC 3000. Work station: Digital DEC 3000. Ionization mode Eleven 70. Column: Capillary column of Fused silica, DB-5 (5% phenyl), 30 m length, 0.32 mm i.d and 0.25 μm thickness. Carrier gas: Helium at 13 psi. Temperature-programming: initial temp. 60 °C for 15 min, 60–220 °C, at a rate of 5 °C/min., final temp. 250 °C for 15 min., ionization voltage: 70 eV. The identification of the compounds was based on comparison of the retention times and mass spectra with corresponding data components of references oils and pure compounds whenever possible. Mass spectra were compared with those of the published data by Adams (1989). Qualitative determination was based on peak area measurements.

Preparation of the emulsions

Emulsions of rosemary (*R. officinalis* L.) and sweet marjoram (*M. hortensis* Moench) were prepared respectively by mixing five drops of Triton-X 100 emulsifier with 2 ml of oil plus an amount of water to obtain 2% concentration of the oil emulsion. A series of dilution i.e. 1, 0.5, 0.25 and 0.125% were prepared from the stock 2% solution using water.

Maintenance of mite stock cultures

The stock cultures of two spotted spider mite *T. urticae* were collected from infested lima bean (*Phaseolus vulgaris* L.) in the laboratory at N.R.C. Cairo. The citrus brown mite, *E. orientalis,* was obtained from cultures maintained on sweet potato. The mites were reared in a controlled climate room at 25–27 °C and 60± 5% R.H.

Pots of lima bean seedlings cultivated in soil were placed in center of the cage (measured 70 x 40 x 60 cm). A continuous light source of (60 watt) flourcent was provided. Large numbers of *T. urticae* were placed on leaf of the lima bean.

Treatment

REPELLENCY TEST PROCEDURE FOR ADULT FEMALES OF *T. URTICAE* AND *E. ORIENTALIS*

Raspberry leaf discs were placed with the lower surface upwards in petri-dish lined with moist cotton wool. Half of each disc was painted with the proper concentration, while the other half treated with water only. Adult females were placed on the midrib and observations on repellency and oviposition were taken after 24, 48 and 72 h, respectively. Each treatment comprised 5 replicates, each replicate contained 10 females. The repellency (mites which had left the discs were considered as repelled) was calculated according to Lwande et al. (1985).

TOXICITY AND BIOLOGICAL EFFECTS OF ROSEMARY AND SWEET MARJORAM ON ADULT FEMALES OF *T. URTICAE* AND *E. ORIENTALIS*

Adult females of the same age of both species were placed individually on raspberry leaf discs treated with different concentrations of both oils. Twenty replicate leaf discs were used and a similar number of discs treated with water only as control. The total number of eggs laid on the treated discs were recorded over a period of 10 days. The mortality of both tetranychid mites was also recorded.

All the experiments reported herein were carried out in the laboratory at 27±2 °C and 70–75 % R.H.

Statistical analyses were carried out by using "F" test .

Results and Discussion

Chemical constituents of rosemary and sweet marjoram oils

GC-MS and GLC analysis of *R. officinalis* and *M. hortesis* essential oils, detected the presence of the following compounds:

1. Hydrocarbon terpenes.
2. Oxygenated terpenes.
3. Hydrocarbon sesquiterpenes.
4. Oxygenated sesquiterpenes.

One can see in *Tables 1, 2, 3* and *Figs. 1, 2* the identification of forty-four components which represent about 90.5% of the total rosemary oil. The oil is characterized by the dominant occurrence of oxygenated hydrocarbon terpenes which contributed with 79.922% of oil content. As assessed from the total peak area, cineole represented the major compound (ca 22%), followed by camphor (9.77%) and linalool (5.01%). The same tables indicated also that the oxygenated sesquiterpenes represented the least content value (2.94%) only.

Table 1

The content of the terpene hydrocarbon compounds in sweet marjoram and rosemary oils

Sweet marjoram oil			Rosemary oil		
Peak No.	Compounds	%	Peak No.	Compounds	%
1	Tricyclene	0.003	1	α-Pinene	5.006
2	α-Thujene	2.054	2	Camphene	1.886
3	α-Pinene	1.126	3	β-Pinene	0.526
4	Camphene	0.046	4	Myrecene	1.762
5	Sabinene + β-Pinene	13.999	5	2-Carene	1.500
6	Myrecene	1.251	6	β-Phellandrene	0.168
7	α-Phyllendrene	0.574	8	γ-Terpinene	0.516
8	3-Carene	0.499	9	Terpinolene	0.700
9	α-Terpinene	2.260		Unknown	1.128
10	p-Cymene	7.802		Total	13.192
11	Limonene	1.778			
12	γ-Terpinene	21.456			
	Unknown	0.035			
	Total	52.883			

Table 2

The content of the oxygenated terpene compounds in sweet marjoram and rosemary oils

Sweet marjoram oil			Rosemary oil		
Peak No.	Compounds	%	Peak No.	Compounds	%
13	cis-p-Menth-2-en-1-ol	1.040	7	Cineole	21.957
14	Terpinolene	0.692	10	Fenchone	0.260
15	trans-p-Menth-2-en-1-ol	3.054	11	Linalool	7.433
16	Linalool	0.566	12	Camphor	9.771
17	cis-Sabinene hydrate	0.241	13	Isoborneol	6.009
18	Citronellal	4.624	14	Borneol	6.915
19	Borneol	0.711	15	Terpinene-4-ol	0.120
20	Terpinene-4-ol	8.246	16	α-Terpineol	6.402
21	α-Terpineol	6.196	17	Verbenone	7.133
22	Nerol	0.293	18	new-iso-Dihydro carveol	1.220
23	Linalyl acetate	1.836	19	cis-Myrtanol	1.901
24	Bornyl acetate	0.212	20	Carvone oxide	0.266
25	Isomenthyl acetate	2.869	21	Bornyl acetate	2.600
26	trans-Carvyl acetate	0.170	22	Carvacrol	0.038
27	Terpinenyl acetate	0.014	24	Eugenol	0.215
28	Neryl acetate	0.308	25	cis-Carvyl acetate	0.189
30	Geranyl acetate	0.413	28	Methyl eugenol	0.271
	Unknown	4.996	30	Geranyl acetate	0.617
	Total	36.48		Unknown	6.618
				Total	79.922

Table 3

The content of sesquiterpene compounds in sweet marjoram and rosemary oils

Sweet marjoram oil			Rosemary oil		
Peak No.	Compounds	%	Peak No.	Compounds	%
	I–Sesquiterpene hydrocarbon compounds				
29	α-Copaene	0.005	23	D-Elemene	0.113
31	β-Bourbonene	0.016	26	α-Copaene	0.132
32	β-Cubebene	0.025	27	β-Elemene	0.034
33	β-Caryophyllene	3.981	29	β-Caryophyllene	2.170
34	Aromadendrene	0.104	31	α-Humulene	0.453
35	α-Humulene	0.279	32	γ-Muurolene	0.179
36	Germacrene-D	0.021	33	Germacrene-B	0.065
37	β-Bisabolene	2.914	34	α-Longipinene	0.117
38	γ-Cadinene	0.019	35	β-Bisabolene	0.090
39	trans-γ-Bisabolene	0.021	36	γ-Cadinene	0.334
	Unknown	0.642	37	Calaminene	0.031
	Total	8.027	38	Trans-γ-Bisabolene	0.053
				Unknown	0.680
				Total	4.451
	II–Oxygenated sesquiterpene compounds				
40	Germacrene-4-ol	0.028	39	Nerolidol	0.013
41	Spathulenol+Caryophyllene oxide	1.048	40	Spathulenol	0.597
42	Globulol	0.150	41	Caryophyllene oxide	0.036
43	Viridiflorol	0.140	42	Tau-Cadinol	0.194
44	Tau-Cadinol	0.138	43	α-Cadinol	0.271
45	α-Cadinol	0.043	44	Epi-α-Bisabolol	0.215
46	Epi-α-Bisabolol	0.003		Unknown	1.109
	Unknown	1.061		Total	2.435
	Total	2.610			

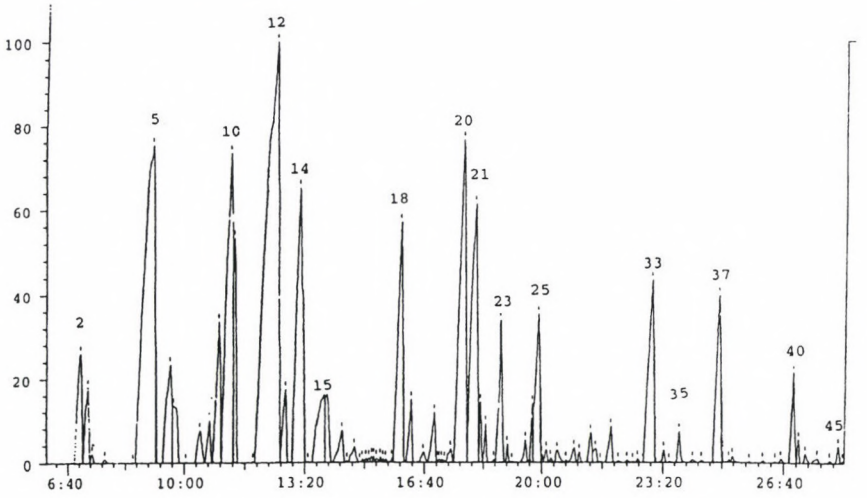


Fig. 1. Chromatogram of marjoram oil

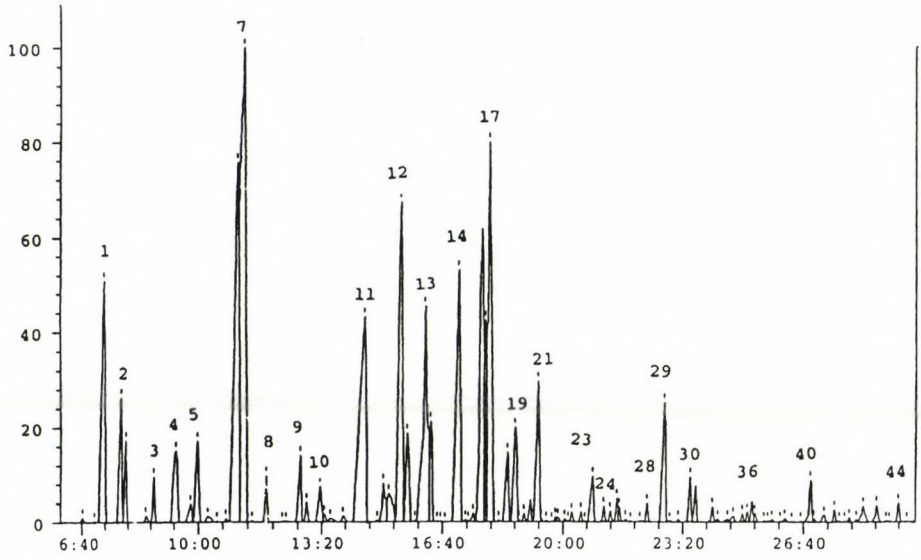


Fig. 2. Chromatogram of rosemary oil

Data in *Tables 1, 2, 3* show that forty-six compounds of sweet marjoram could be identified representing about 93.3% of the total content. It is evident that about 53% of the total oil consisted of terpene hydrocarbons with γ-terpinene accounting about 21.5% of total oil, sabinene + β-pinene and p-cymene represented about 14% and 7.8% of total oil content, respectively. The oxygenated terpenes contributed with 36.48% of the oil content, terpineol-4 and α-terpineol represented about 8.2% and 6.2% of the total oil content, respectively. On the other hand sesquiterpenes and oxygenated sesquiterpenes represented about 8% and 2.6% of the oil content respectively.

Repellency and oviposition deterrence

In *T. urticae,* percent repellency gradually increased with both essential oil concentrations *(Table 4).* On the contrary, rosemary oil proved to be completely deterrent for *E. orientalis* even after 72 h, of treatment and at the lowest concentrations *(Table 5).* Repellency of sweet marjoram oil decreased at 0.125% conc. in comparison with other concentrations *(Table 5).* As for oviposition behavior [Oviposition deterrent indices (ODI) as defined by Lundgren 1975] in case of *E. orientalis,* both oils hindered adults to lay eggs except at 0.125% conc. of sweet marjoram *(Table 5).* The average number of eggs laid by females of *T. urticae* after 72 h of treatment varied according to concentrations *(Table 4).* Mansour et al. (1986) found that bean leaf discs sprayed with concentrations of the acetonic solutions of the rosemary oil from 0.1% to 2% caused mortality and induced repellency to *T. cinnabarinus* (Boisd) within 48 h of placing adult females on the discs and consequently egg-laying was reduced. Mazeed (1987) demonstrated that marjoram oil form

(Origanum majorana) was effective for controlling the spread of *Acaropis woodi* (Rennie) to young workers introduced to infested colonies. Both rosemary and sweet marjoram oils gave 100% repellency to *T. urticae* at 10% level of concentration (Sawires et al., 1988).

The varying effect of the different oils may be due to the varying constituents of oxidative substances within the solid part of the volatile oil.

Table 4

Relative distribution and oviposition of *T. urticae* on treated leaf discs with rosemary and sweet marjoram oils

% concentrations	% distribution on treated part after			% repellency	Avg. No. of eggs/female after 72 h		ODI
	24 h	48 h	72 h		T	C	
				Rosemary			
2	15	20	30	91	0.15	1.65	83.33
1	15	25	30	89.5	0.2	1.9	80.95
0.5	25	30	35	82.1	0.35	1.95	69.57
0.25	35	35	50	67.3	0.9	2.75	50.68
0.125	40	50	80	46.6	1.55	2.9	30.34
				Sweet marjoram			
2	5	5	5	100	0	0.7	100
1	10	10	15	100	0	0.7	100
0.5	15	20	25	92.5	0.2	2.65	85.96
0.25	15	25	30	85.5	0.4	2.75	74.60
0.125	20	25	40	69.6	0.85	2.8	53.42

C = control; T = treated

Table 5

Relative distribution and oviposition of *E. orientalis* on treated leaf discs with rosemary and sweet marjoram oils

% concentrations	% distribution on treated part after			% repellency	Avg. No. of eggs/female after 72 h		ODI
	24 h	48 h	72 h		T	C	
				Rosemary			
2	0	0	0	100	0	0	0
1	0	0	0	100	0	0.25	100
0.5	0	0	0	100	0	0.35	100
0.25	0	0	0	100	0	0.45	100
0.125	0	0	0	100	0	0.45	100
				Sweet marjoram			
2	0	0	0	100	0	0.65	100
1	0	0	0	100	0	1.25	100
0.5	5	0	5	100	0	1.45	100
0.25	5	5	5	100	0	2.7	100
0.125	25	25	25	90.8	0.25	2.7	83.05

C = control; T = treated

Concentration effects on oviposition and mortality

Significant reduction in the total number of eggs laid by both tetranychid mites were recorded during 10 days period for all concentration tested for both essential oils *(Table 6).* Fecundity was severely reduced as the concentration of both oils increased. Previous studies have shown that the essential oils of *Callistemon lanceolatus* DC. and

Table 6

Effect of rosemary and sweet marjoram oils on reproduction and mortality of *T. urticae* and *E. orientalis*

Conc. %	Rosemary		Sweet marjoram oil	
	Total No. of eggs/female/10 days**	% corrected mortality after 10 days*	Total No. of eggs/female/10 days**	% corrected mortality after 10 days*
		T. urticae		
2	1.75	53.85	0.25	90.91
1	2.10	15.38	3.25	27.27
0.5	6.50	8.33	4.80	25.00
0.25	6.90	7.69	10.20	23.10
0.125	13.10	7.14	17.15	21.43
Control	58.5	–	58.5	–
		E. orientalis		
2	0	100	0	100
1	0	100	0.20	100
0.5	1.10	71.43	0.50	92.86
0.25	1.50	60.00	1.90	86.67
0.125	2.35	60.00	2.30	80.00
Control	15.4	–	15.4	–

*Counts according to Abbott's Formula (1925).
**Highly significantly at $P < 0.01$
[statistical analysis based on Least Significant Difference (L.S.D. test)]

Cotula cinerea L. had deterrent effects on females of *T. urticae* and spraying females with LC_{50} significantly reduced its fecundity (Ibrahim and Amer, 1992; Ibrahim et al., 1993). However, percentage mortality was significantly concentration dependent. In case of *E. orientalis* at 2% and 1% conc. it was high with both oils and reached 100% with *T. urticae* the trend was similar albeit at a slightly lower rate increasing from 7.14 and 21.43% to 53.85 and 90.91% with rosemary and sweet marjoram, respectively. The results clearly depended on the mite used for experiments, as marked differences in mortality were recorded. Amer et al. (1993) reported that the orange peel oil showed a remarkable deterrent effect with *E. orientalis,* whereas the essential oil of lemon grass in addition to the orange peel oil was toxic to females and eggs of *T. urticae* and *E. orientalis.* Regnault and Hamraoui (1995) suggested that lipid as well as non-lipid allelochemicals, such as phenolics, or non-protein amino acids or flavonoids, may be involved in the toxicity of extracts of aromatic plant *R. officinalis* and *Eucalyptus globulus* to *Acanthoscelides obtectus.* El Gengaihi et al. (1992) confirmed the role of volatile oil as having antifeedant and growth

inhibiting effects of *Vitex agnus costus* on *Spodoptera littoralis* larva. Rosemary and sweet marjoram oils were promising for possible use against *T. urticae* and *E. orientalis.* It is worth mentioning that oils with such properties will not disturb ecosystems and consequently, will not cause outbreaks of new pests, as persistent insecticides are apt to do.

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A Comparative Study of the Effect of Some Mineral and Plant Oils on the Two Spotted Spider Mite *Tetranychus urticae* Koch (Acari: Tetranychidae)

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The direct toxicity of some mineral and plant oils to the eggs and females of the two spotted spider mite *Tetranychus urticae* Koch was tested. KZ oil was toxic to the egg stage compared to adult female. In contrast, Natur'l oil has a close toxic effect for both stages of *T. urticae*. Bio-dux oil was proved to be toxic to adult female and relatively in toxic to egg stage. Females of *T. urticae* suffered a depression in reproduction and shortened longevity when kept on plant leaves previously treated with different oils. Laboratory studies indicated that the vegetable oil Natur'l oil was effective on all biological aspects of *T. urticae* studied.

Keywords: Acari, mineral oils, plant oil, *T. urticae*.

The two spotted spider mite, *Tetranychus urticae* Koch, has been evaluated as a potentially serious pest of many agricultural crops (Jeppson et al., 1975; Helle and Sabelis, 1985). Mineral spray oils of various qualities have traditionally been used as curatives to down small insect and mites (Hely et al., 1982; Davidson et al., 1991). These oils are cheaper and less phytotoxic than their predecessors (Riehl, 1969, 1981; Beattie et al., 1989), as well as, no empirically demonstrated resistance by pests. Modern petroleum spray oils meet the criteria better than most alternatives. They are ideally suited to IPM and organic farming (Davidson et al., 1991) and resistance is unlikely (Beattie and Smith, 1996).

A few published data on the susceptibility of mite pests to oils are limited to citrus red mite *Panonychus citri* (McGregor) (Riehl et al., 1958; Trammel, 1965; Herron et al., 1995), the European red mite *Panonychus ulmi* (Kock) as well as the spider mites *Eutetranychus orientalis* (Klein) and *T. urticae* (Osman, 1997; Herron et al., 1998).

The immediate goal of the present study is to evaluate the efficacy of some mineral and plant oils that can be used in integrated control of *T. urticae,* the main acarine pest of vegetables.

Materials and Methods

Mineral oils

Sol EC: [it is a solar cut petroleum oil fraction prepared as emulsifiable concentrate contained 96.62% (v./v.) base oil], Capl-1: (it is sulfonated solar oil prepared as emulsifiable concentrate, registered by Central Agriculture Pesticides Laboratory), Capl-2: [lubrication cut of petroleum oil fraction prepared as emulsifiable concentrate

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contained mineral oil 96.62% (v./v.)], KZ oil: [prepared as emulsifiable concentrate contained 95% Paraffin oil (w./w.)], Citrole: (made from a biodegradable paraffin as an emulsifiable formulation permitting ease of mixing with water), Kemesol: [local mineral oil prepared as emulsifiable concentrate contained 95% (v./v.) base oil], Bio-dux: [15% potassium oleate (150 g/kg at 20 °C)].

Vegetable oil

Natur'l oil: [Natural vegetable oil 93% (w./v.)].

COLLECTION AND MAINTENANCE OF MITES

The two spotted spider mite *T. urticae* were collected from infested cucumber leaves from plantations at Giza and reared on Lima bean [*Phaseolus vulgaris* (L.)] under standard laboratory conditions (25±2 °C; 65±5% R. H.).

Toxicity bioassay

DIRECT EFFECT ON ADULT FEMALES

Adult females of *T. urticae* were confined on the lower surfaces of detached raspberry leaves (3 cm in dia.) while the upper surfaces were placed on cotton saturated with water. Mites were sprayed with different oils using a glass atomizer.

For each oil, 5 concentrations were tested in addition to a control. A total of 30 adult female spider mites were used in each treatment and each experiment was repeated 3 times. Mites that did not walk when gently prodded were counted as dead. Mortality was recorded 48 h after application.

DIRECT EFFECT ON EGG STAGE

The direct effect on egg stage was studied by confining to females on leaf discs for 24 h, then removed. These discs were sprayed with deposited eggs (24 h old) in a series of concentrations for 50 sec., and allowed to dry. Observations on viability were made twice a day. Eggs that had not detached after 7 days since oviposition were considered non-viable. The experiment was repeated 3 times. A control was included in each experiment.

Corrected mortality counts according to Abbot's formula (1925), then submitted to probit analysis using Finney (1952)

$$\text{Relative toxicity} = \frac{LC_{50} \text{ of the most active oil} \times 100}{LC_{50} \text{ of certain oil}}$$

Biological Tests

BIOASSAY FOR RESIDUAL ACTIVITY

In a third series of experiments, the residual activity of each oil was investigated. Leaf discs were dipped for 60 sec in the LC_{50} of each of the aforementioned oils. The leaf discs were placed on a layer of wet cotton wool on top of a sponge in a tray with a sufficient amount of water to keep the sponge wet. One newly emerged adult female spider mite was placed on each disc. Untreated discs were used as a control. Each treatment was replicated 20 times.

Statistical analysis were carried out using F test. Observations on different biological aspects were recorded daily.

Results and Discussion

Toxicity to adult females

The data obtained in *Table 1* show that Citrole oil was the most toxic to adult female, while KZ oil was the least. The LC_{50} values of the tested oils toxicity were in descending order of effectiveness as follows: Citrole, Kemesol, Natur'l oil, Capl-1, Sol, Capl-2, Bio-dux and KZ oil. Herron et al. (1998) reported that the petroleum spray oil C 23 was generally more efficacious on adult female *P. ulmi* and *T. urticae* than C 21 oil. Research carried out by Osman (1997) indicated that the petroleum oils (Albulium 80% EC, KZ 95% EC, Shokrona super EC 95%) were more toxic to adult females of *E. orientalis* than that of *T. urticae.*

Table 1

Toxicity of some mineral and plant oils against adult females *T. urticae*

Materials	LC_{50}	LC_{90}	Slope	Relative toxicity at LC_{50}	Relative toxicity at LC_{90}
Citrole	0.975	9.598	1.29	100	59.64
Kemesol	1.055	5.724	1.77	92.42	100
Natur'l oil	2.055	12.85	1.61	47.45	44.54
Capl-1	3.195	10.27	2.52	30.52	55.74
Sol	3.878	10.10	3.07	25.14	56.67
Capl-2	4.336	8.628	4.28	22.49	66.34
Bio-dux	4.812	9.378	4.42	20.26	61.04
KZ oil	5.731	73.89	1.15	17.01	7.75

Toxicity to egg stage

KZ and Citrole oils have been shown to be toxic oils on egg stage of *T. urticae (Table 2),* Tested oils can be arranged according to their LC_{50} values for egg stage in the following descending order of effectiveness: KZ, Citrole, Sol, Capl-1, Kemesol, Capl-2, Natur'l oil and Bio-dux. Results from *Fig. 1* show that egg stage more sensitive to different oils than adult stage, except for the Bio-dux which was toxic to adult stage than egg stage (LC_{50} = 4.812 and 11.46% respectively). Results indicated also, that KZ oil was more toxic to egg stage than to adult stage (LC_{50} = 0.20 and 5.731% respectively).

It is worth mention also that the toxicity effect of Kemesol oil and Natur'l oil on both stages were very close (LC_{50} = 1.055 and 0.638%; and LC_{50} = 2.055 and 1.494% respectively). Osman (1997) demonstrated that the response of egg stage of *T. urticae* and *E. orientalis* to the mineral oils varied according to their age.

Table 2

Toxicity of some mineral and plant oils against egg stage of *T. urticae*

Materials	LC_{50}	LC_{90}	Slope	Relative toxicity at LC_{50}	Relative toxicity at LC_{90}
KZ oil	0.20	0.642	2.52	100	78.66
Citrole	0.232	0.505	3.79	86.21	100
Sol	0.579	1.191	4.09	34.54	42.40
Capl-1	0.618	1.846	2.69	32.36	27.36
Kemesol	0.638	1.614	3.17	31.35	31.29
Capl-2	0.878	3.513	2.13	22.78	14.38
Natur'l oil	1.494	12.36	1.40	13.39	4.09
Bio-dux	11.46	32.26	2.85	1.75	1.57

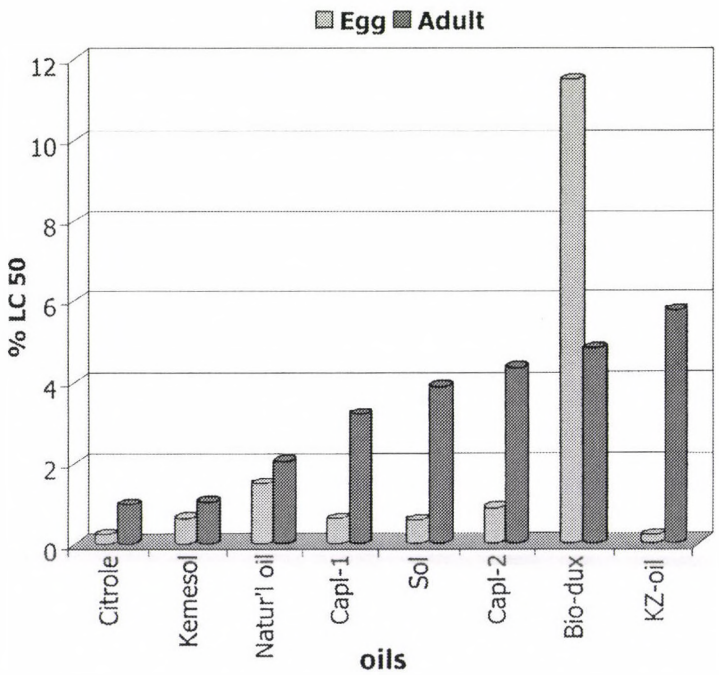


Fig. 1. Toxicity of some mineral and plant oils against egg stage and adult females of *T. urticae*

The greater susceptibility to the petroleum spray oils of *P. ulmi* compared to *T. urticae* was reported by Herron et al. (1998) as well as adult female of *P. citri* were significantly more susceptible than adult female *T. urticae* (Herron et al., 1995). They suggested that the different responses might be related to variation in the sizes and distribution of dorsal setae and the structure and functioning of their respiratory systems.

Biological tests

BIOASSAY FOR RESIDUAL ACTIVITY

It is clear from *Table 3* that the mineral and plant oils treatment used, prolonged the preoviposition period and showed highly significant effects at Natur'l oil, Capl-1 and Bio-dux oils. The incubation period was affected also and showed highly significant effects at citrole, Kemesol and Natur'l oils. On the other hand, the LC_{50} treatment of each oil used, shorted the female longevity and the female fecundity was significantly reduced. The vegetable oil, Natur'l oil was generally highly efficacious on all biological aspects of the adult female *T. urticae (Table 3).*

Table 3

Effect of tested oils (LC_{50} conc.) on different biological aspects of *T. urticae*

Materials	Biological aspects, periods in days			
	Pre-oviposition ±S.E.	Incubation ±S.E.	Female longevity ±S.E.	Total No. of eggs/female ±S.E.
Citrole	1.4 ±0.11	4.05±0.14**	4.2 ±0.55**	4.95±0.79**
Kemesol	1.35±0.11	3.6 ±0.11**	3.8 ±0.42**	7.9 ±0.78**
Natur'l oil	2.2 ±0.27**	3.55±0.21**	5.4 ±0.54**	1.9 ±0.31**
Capl-1	2.05±0.29**	2.8 ±0.17	5.5 ±0.53**	8.7 ±1.82**
Sol	1.4 ±0.18	2.55±0.17	2.8 ±0.14**	2.05±0.23**
Capl-2	1.5 ±0.20	2.75±0.18	4.1 ±0.63**	3.9 ±0.63**
Bio-dux	2.1 ±0.19**	2.5 ±0.15	5.65±0.47**	2.3 ±0.48**
KZ oil	1.7 ±0.21	2.85±0.15	4.2 ±0.51**	1.7 ±0.24**
Control	1.3 ±0.11	2.5 ±0.11	18.9 ±0.37	74.9 ±0.93
L.S.D. 0.05	0.543	0.440	1.341	2.328
L.S.D. 0.01	0.717	0.581	1.769	3.072

**High significant ≤ 0.01%

Osman (1997) demonstrated that petroleum spray oils, Albulium 80% EC, KZ 95% EC, Shokorana super 95% EC and Shokorana 95% EC were significantly reduced the fecundity of both spider mites *T. urticae* and *E. orientalis.* The studies by Rae et al. (1999) on citrus psylla have shown that 0.5% petroleum oil sprays deter oviposition, and kill eggs and nymphs. They also showed that these sprays are as effective as omethoate and diflubenzuron (an IGR), both of which are currently used to control the pest in China. Rizk et al. (1999) reported the succes of the petroleum oils, Capl-1, Capl-2 and Sol-EC against adult of *Tetranychus letoralis* on cotton plant.

Conclusion

Pesticides must be carefully chosen and should only be used when natural enemies fail to keep pest population below economic thresholds. Unlike insecticides, oils are not acutely poisonous and generally kill by suffocation (Ebeling, 1959; Riehl, 1967).

The ability of the two spotted spider mite to retract its stylophore and restrict air movement into the peritremes is thought to be related to its tolerance to toxic gases and its ability to withstand desiccation (Jeppson et al., 1975; Helle and Sabelis, 1985). The information presented here is based on laboratory experiments, and indicated that the vegetable oil (Natur'l oil), had a close toxicity effect on both stages, eggs and adult female as well as its effect on the biological aspects is promising. The results of the present study indicated that different mineral oils can be effective on the pest *T. urticae.*

Further field studies will indicate the possibility for using mineral and plant oils to control the pest mite in the field. According to Davidson et al. (1991), use of petroleum spray oils in low-volume sprays at lower rates per hectare than used conventionally, also reduce risk of phytotoxicity.

Much information is needed on the impact of different mineral and plant oils on natural enemies (insects and mites) associated with spider mites in the field.

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Effects of Diet on the Biology and Life Tables of the Predacious Mite *Agistemus exsertus* (Acari: Stigmaeidae)

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The biology of *Agistemus exsertus* Gonzalez was studied using two different insect eggs, *Ephestia kuehniella* Zeller and *Parlatoria zizyphus* (Lucas) as the food source. The development was faster and reproduction was higher when *A. exsertus* fed on eggs of *E. kuehniella*. A total of 97.78 and 75.27 eggs per female were obtained when eggs of *E. kuehniella* and *P. zizyphus* were provided respectively. A diet of *E. kuehniella* (eggs) provided the greatest female longevity and mean total fecundity which resulted in the higher net reproductive rate (Ro) value (61.25), intrinsic rate of natural increase (rm = 0.196) and finite rate of increase (rm = 1.22) per day for *A. exsertus*. A diet of *P. zizyphus* (eggs) resulted in close values of T = 21.70; rm = 0.174; $e^{r}m$ = 1.19. The sex rate of the progeny was strongly female biased (female/total = 0.72 and 0.66) when both eggs of *E. kuehniella* and *P. zizyphus* were provided.

Keywords: Acari, *Agistemus exsertus, Ephestia kuehniella,* life table, *Parlatoria zizyphus.*

Mites of the family Stigmaeidae are live in the soil, stored products and on plants and are usually predators of mites. A few prey on scale insects or parasitize flies (Swift, 1987) and others feeds on mosses (Gerson, 1972). The stigmaeid mite *Agistemus exsertus* Gonzalez has most commonly been recorded in association with spider mites and scale insect in Egypt. The predacious mite *A. exsertus* feeds not only on tetranychid mites and pollen grains, but also on eriophyid and tenupalpid mites (Soliman et al., 1976; Hanna et al., 1980; Oamen, 1982; El-Bagoury and Reda, 1985; Santos and Laing, 1985).

A. exsertus is documented by many authors as an egg predator of tetranychid and tenupalpid mites as well as the tobacco white fly *Bemisia tabaci* (Genn.) (El-Badry et al., 1969; Hafez et al., 1983; El-Bagoury et al., 1989; Abou-Awad and El-Sawi, 1993). It was found that the egg stage was a more favorable food than immature stages. The major objective of this study of life tables was undertaken to investigate the nutritional value of an egg stage of both the stored product moth *Ephestia kuehniella* Zeller and the scale insect *Parlatoria zizyphus* (Lucas) to the predacious mite *A. exsertus,* in order to make a preliminary evaluation to their potential role as food.

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Materials and Methods

Maintenance of mite stock cultures

Adult females of *A. exsertus* used in this study were collected from heavily infested Ploughiman's spikenard plant at Faywam Province, Egypt in 1999; and reared on eggs and immature stages of the two spotted spider mite *Tetranychus urticae* Koch at 27 °C and 70 ± 75% R.H.

Diets

Two diets were evaluated for their effect on development survival, oviposition and life table parameter:

1. Eggs of the stored product moth *E. kuehniella* were obtained from stock culture kept at the laboratories of N. R. C. for many years.
2. Eggs of the scale insect *P. zizyphus* were collected from commercial citrus orchards.

Effects of diet on development and survival

The rearing arena (3 x 3 cm) of excised raspberry leaves, placed on saturated cotton in plastic petri dishes, were used to confine the predator. A strip of moistened absorbent cotton was placed around the outside edge of the leaves. A single newly deposited egg was transferred to each arena and the newly hatched larvae were supplied with the food resource to be evaluated. All handling of mites and host eggs was performed with a very fine, moistened, squirrel hairbrush. Arenas were examined daily and predator development and survival was recorded. Prey eggs consumed were replaced daily by fresh eggs to maintain an ample food supply.

Effects of diet on longevity, fecundity and life table parameters

Newly emerged female, mated, confined individually on test arenas, along with the food to be tested. A few strands of cotton wool were provided as an ovipositor site on each arenas. Oviposition and survival were recorded as well as stigmaeid eggs were removed daily for sex determination. An egg diet was considered consumed whenever a depression of the chorion was noticed after the predator attack, indicating that at least some feeding had occurred. As piercing the chorion and feeding for only a few seconds resulted in the eventual collapse of an egg.

Twenty five eggs of *A. exsertus* in each experiment were observed daily, and each experiment was repeated twice.

Life table parameters as defined by Birch (1948) were calculated according to a BASIC computer program (Abou-Setta et al., 1986).

Results and Discussion

Effects of diet on development and survival

Individuals of *A. exsertus* successfully developed from larva to adult when fed on eggs of either of *E. kuehniella* and *P. zizyphus (Table 1).* The developmental period was significantly rapid on eggs of *E. kuehniella* than on *P. zizyphus* (significant at 5% level). Several authors showed that an egg stage of *P. zizyphus* provides enough nutrition for various predacious mites of the family Phytosiidae to complete development (Aly, 1994; Abou-El-ella, 1998; Momen, 1999; Momen and Hussein, 1999). Ragusa and Tsolakis (1994) reported that when eggs of *E. kuehniella* were offered to the predacious phytoseiid mite, *Amblyseius andersoni* (Chant), the percentage of individuals attaining adulthood was 8%, as well as the young stages were in a very poor condition, thin, not mobile. The fraction of *A. exsertus* eggs reaching maturity was similar with both diets (0.88).

Table 1

Comparative duration ($x \pm$ S.E.) of female of *A. exsertus* at 27 °C on test food

Developmental stages	*P. zizyphus* (eggs)		*E. kuehniella* (eggs)	
Egg	2.0 ± 0.0		2.0 ± 0.0	
Larva	2.3 ± 0.1	a	1.6 ± 0.1	b
Protonymph	4.6 ± 0.2		4.1 ± 0.2	
Deutonymph	6.1 ± 0.2	a	5.1 ± 0.2	b
Life cycle	15.0 ± 0.4	a	13.0 ± 0.3	b
Preoviposition period	2.6 ± 0.1		2.1 ± 0.1	
Oviposition period	18.4 ± 0.9	a	25.4 ± 1.0	b
Adult longevity	23.6 ± 0.9	a	31.2 ± 0.8	b
Life span	38.6 ± 1.1	a	44.2 ± 0.8	b

Different letters in a horizontol column denote significant differences (F test. $P \leq 0.05$).

Effects of diet on longevity, fecundity and life table parameters

The preoviposition period was almost the same with the two different insect diets *(Table 1). A. exsertus* females reared on *E. kuehniella* eggs had a significantly longer oviposition period and a significantly greater total fecundity than *A. exsertus* females reared on *P. zizyphus* eggs *(Tables 1* and *2).* Higher oviposition rates were associated with longer oviposition period and lower oviposition rates were associated with shorter oviposition period. Mean total fecundity was 97.78 eggs/ ♀ over a mean oviposition period of 25.4 days for *A. exsertus* reared on *E. kuehniella* eggs compared to 75.27 eggs/ ♀ over a mean oviposition period of 18.7 days for *A. exsertus* reared on eggs of *P. zizyphus.* Longevity of females *A. exsertus* was significantly different when reared and maintained on eggs of *E. kuehniella* eggs compared to eggs of *P. zizyphus.* Longevity of *A. exsertus* adult females ranged from 24 to 36 days ($\bar{x}$ = 31.21 days) when reared on a diet of *E.*

Table 2

Effect of diet on the life table parameters of *A. exsertus* at 27 °C

Parameters	*P. zizyphus* (eggs)	*E. kuehniella* (eggs)
Mean total fecundity (eggs/ ♀)	75.27	97.78
Net reproductive rate (Ro)	43.71	61.25
Generation time (days) (T)	21.70	20.99
Intrinsic rate of increase (rm)	0.174	0.196
Finite rate of increase ($e^r m$)	1.19	1.22
Sex ratio (females/total)	0.66	0.72

kuehniella compared to a range of 18 to 30 days ($x^- = 23.6$ days) when reared on a diet of *P. zizyphus* eggs *(Table 1).* As noted by some authors that maximum fecundity of 134.5, 97.0 and 90.50 eggs/female of *A. exsertus* was recorded at 27–29 °C, respectively, when fed on eriophyid mite *Eriophyes dioscoridis* Soliman and Abou-Awad, artificial diet composed (milk + yeast + amino acids + sugar) and eggs of *T. urticae* (El-Bagoury and Reda, 1985; Reda, 1990; Abou-Awad and El-Sawi, 1993). *A. exsertus* has been reported to feed on dates pollen and the acarid mite *Tyreophagous casei* Oudemans and the daily rate of reproductivity averaged 3.0 eggs / ♀/ day for both diets (Abou-El-ghar et al., 1969; Rasmy et al., 1987). The reproductive rate of *A. exsertus* feed on eggs of *P. zizyphus* in this study, fall within the range of the predator known to feed on eggs of *Eutetranychus orientalis* klein, *Brevipalpus plucher* (C. and F.) and *Bemisia tabaci* (Genn.), respectively (Abou-Awad and El-Sawi, 1993). Eggs of *P. zizyphus* were unsuitable for the predator *Amblyseivs lindquisti* Schuster and Pritchard feeding and reproduction (Momen, 1999), while the rate of reproduction of *Typhlodromus transvaalensis* (Nesbitt) was very low (1.54/ ♀) when fed on the same diet. No oviposition occurred or it was unsatisfactory when *A. andersoni* fed on eggs of *E. kuehniella.*

Life table parameters

Mean generation time (T) of *A. exsertus* did not vary much between two egg diets *(Table 2).* The net reproductive rate (Ro), which is a product of mean total fecundity, survival rate and sex ratio, followed the same pattern as total fecundity. The higher (Ro) value of 61.25 expected females per female was obtained with a diet of *E. kuehniella* eggs. The lower (Ro) value of 43.71 expected females per female was obtained with a diet of *P. zizyphus* eggs *(Table 2).* The intrinsic rate of increase (rm) and, subsequently, the finite rate of increase ($e^r m$) were relatively higher (0.196 and 1.22 respectively) when individuals were fed *E. kuehniella* eggs and lower when fed *P. zizyphus* eggs (0.174 and 1.19). The higher (rm) for *A. exsertus* reared on eggs of *E. kuehniella* could be attributed to differences in nutritive quality. The consumption of more suitable prey (i.e. egg/ larva/nymph), favouring a high conversion of food into egg biomass, determined higher intrinsic rates of increase (Sabelis, 1985; Bruce-Oliver and Hoy, 1990).

Comparison of life table parameters of *A. exsertus* on tetranychoid egg diets or eggs of whitefly (Abou-Awad and El-Sawi, 1993), may be possible because they were conducted under similar conditions (temperature and technique). The intrinsic rate of increase is higher on diet of *E. kuehniella* eggs than those of tetranychoid and whitefly eggs. The (Ro) value of *A. exsertus* fed eggs of *E. kuehniella* fall within the range of the predator known to control *T. urticae* eggs, while the (Ro) value of the predator fed eggs of *P. zizyphus* fall whithin the close range of predator known to control eggs of *E. orientalis, B. pulcher* and *B. tabaci.*

This study indicated clearly that the pyralid eggs of *E. kuehniella* favored the full expression of the reproductive potential of the predacious mite *A. exsertus.*

Conclusions

This study indicated that *A. exsertus* developed and reproduced successfully on either eggs of *E. kuehniella* (Fa. Pyralidae) and *P. zizyphus* (Fa. Diaspididae). The use of life table values to predict potential efficacy of this biological agent for augmentation or classical biological control programs requires caution. In our experiments, varying the egg diets produced substantial differences in life table values, which underscores the importance of the biotic test conditions.

Muma (1971) divided candidate food offered to various female phytoseiids in the laboratory into four categories: optimal, adequate, survival and inadequate foods. According to these criteria, food tested in the present work could be divided as follows: optimal food *(E. kuehniella),* adequate food *(P. zizyphus).* The interest in the stored product moth *E. kuehniella* is due to its possible function as basic diet for the predacious mite *A. exsertus,* which has not been investigated before for family Stigmaeidae.

From this it can be concluded that *A. exsertus* consider as an egg-predator and is promising biological control agent of insect pests such as stored product moth or scale insect (in the present study) and the tobacco whitefly (Abou-Awad and El-Sawi, 1993). I would suggest further studies to develop method for determining predation rates by the predator on both pyralid and diaspidid eggs.

More investigation is needed also on the predatory impact and responses of *A. exsertus* to different insect and mite species (e.g. Thrips–Lepidoptera–Tarsomemid)

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Effects of Forestry Practices on Carabids (Coleoptera: Carabidae) – Implication for Nature Management

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Effects of forestry management were studied in the Szatmár-Bereg Landscape Protection Area (NE-Hungary). Carabid assemblages of forest stands managed by different management techniques (stand put under acorn after clearing the herbaceous and shrub layer, the other prepared for seedlings by grubbing and deep loosening) have been compared with that of a non-managed control stand using pitfall traps. The number of carabid individuals and species has been found to be the highest in the non-managed stand, followed by that of the stand which was put under acorn after clearing the herbaceous and shrub layer. The fewest individuals and species were observed in the stand managed by grubbing and deep loosening. There was no significant difference between the species richness of the control stand and the stand managed by clearing the herbs and shrubs and put under acorn, while both values were found to be significantly higher than that of the stand managed by grubbing and loosening. The composition of the carabid assemblage of the non-managed stand and that of the stand cleared and put under acorn were similar to each other, while the carabid assemblage of the stand managed by grubbing and deep loosening was considerably different from the assemblages of the two above stands. The results suggest that the grubbing and deep loosening management practice completely changes the structure and composition of the carabid assemblage, thus it is not recommended to use in protected areas. Clearing the herbaceous and shrub layer followed by putting under acorn, does not substantially change the structure and composition of the carabid assemblage, so it can be used on protected areas for forestry management.

Keywords: Carabids, forestry practices.

Recently 18.2% of the land surface has been forested in Hungary. However, 75% of these forests is used primarily for economic purposes. In the 20th century the area of forest stands composed of native deciduous trees (e.g. oak, beech, hornbeam etc.) decreased remarkably due to the modern forestry practices, while the area of non-native plantations (conifer, poplar, robinia plantations) expanded exponentially (Mátyás, 1996). In the past 50 years the common forestry plan was that the felled native forest stand was reforested with non-native trees. However, planting of non-native species has drastic effects on the native assemblages through both the direct destruction of their habitats and the alteration of the abiotic and biotic environmental conditions. Therefore, nowadays there has been an increasing demand for the renewing of native forests instead of the establishment of non-native plantations. It is even more important on protected areas (Samways, 1994).

Ground-dwelling beetles are highly sensitive to the changes in the vegetation and the accompanying alteration both in the soil conditions and the microclimate, therefore in the present paper we have studied the reaction of carabid beetles (Coleoptera: Carabidae)

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to the different forestry practices. Several previous studies (Szyszko, 1986; Magura et al., 1997) revealed that non-native reforestation had significant impacts on carabids. Moreover, it has been reported that the clear cutting also damaged the native carabid assemblages (Mader, 1986; Šustek, 1984; Niemelä et al., 1993, 1994). The Hortobágy National Park Directorate launched a research program in the Szatmár-Bereg Landscape Protection Area in the 1990s to investigate the effects of the different forest management practices on the biota and to prepare an ecologically sound forestry practice.

In the present study we examined the impacts of different forestry practices on carabid beetles. Moreover, we studied whether there was a forest practice that did not substantially change the structure of the native carabid assemblages, therefore which can be used on protected areas for management.

Materials and Methods

Study area and sampling

The sampling area was located in the Szatmár-Bereg Landscape Protection Area, in NE-Hungary. In this area the oak-hornbeam forest *(Querco robori-Carpinetum)* is the most extensive forest type. On the research area forest stands managed by different management techniques were studied: (1) a control stand: a 40–50-year-old oak-hornbeam stand without forestry management, (2) a 40–50-year-old oak-hornbeam stand which was put under acorn after clearing the herbaceous and shrub layer in 1996 and (3) a 16-year-old oak-hornbeam which was clear-cut and prepared for seedlings by grubbing and deep loosening.

All of the studied forest stands were located in the Boc-kerek forest (its forest type was *Querco robori-Carpinetum;* its area was approx. 600 ha). Forest stands were at least 250 meter from the boundaries between the forest and the neighbouring open area (grassland and agricultural area), therefore the carabid assemblages of the forest stands can be considered independent from that of the adjacent open area (Digweed et al., 1995). Moreover, forest stands were at least 150 meter far from each other, thus their carabid assemblages can also be regarded as independent assemblages (Digweed et al., 1995).

Beetles were sampled using unbaited pitfall traps containing ethylene-glycol as a killing-preserving solution (Spence and Niemelä, 1994). In each habitat 12 pitfall traps were used during the sampling procedure. The traps (diameter 100 mm, volume 500 ml) were placed in a grid of 3 x 4 traps (2–3 m far from each other) in the interior of each forest stand. Samples were collected monthly from January to October in 1997. For the numerical analyses pooled samples were used since several authors (Baars, 1979; Loreau, 1992) showed that the total capture of a species over the whole sampling period gave an estimate of the ecological importance of each species in a habitat in case the sampling period was long enough to cover the most of the beetles' activity period.

Data analyses

Observed number of individuals, number of species and expected number of species (ES(m)-diversity) were compared among the studied habitats. ES(m)-diversity eliminates the effect of sample size on species number. ES(m)-diversity (sometimes mentioned as rarefaction) is a statistical method for estimating the number of species expected in a random subsample drawn from a larger sample (Hurlbert, 1971; Smith and Grassle, 1977; Tóthmérész 1995). Thus, ES(m)-diversity gives the number of species present when m individuals are drawn at random from the population:

$$ES(m) = \sum_{i=1}^{S} \{1 - (1 - p_i)^m\}$$

where p_i is the abundance of the i-th species of the assemblage and m is an integer, but real values make mathematical sense.

The resulting value can be interpreted as a "diversity index" because the method takes into account both the species richness and the relative abundance. ES(m)-diversity is an accepted and reliable statistical method studying carabid species richness (Niemelä et al., 1990, 1992b, 1993, 1994; Niemelä and Halme, 1992; Cárcamo et al., 1995). We estimated the number of species in a subsample of 380 individuals from each habitat using ES(m)-diversity. Statistical tests of the difference in the ES(m)-diversity of the compared stands were based on the normal approximations published by Tong (1983). The calculations were performed by the DivOrd package (Tóthmérész, 1993a).

Similarity of the carabid assemblages was calculated by the Bray–Curtis index of similarity, which is a generally used measure of compositional similarity (Ludwig and Reynolds, 1988; Niemelä et al., 1993). The similarity structure was displayed by cluster analysis using the Ward-Orlóci fusion method (Gauch, 1986). The NuCoSA package (Tóthmérész, 1993b) was used during the calculation.

Results

A total of 1697 individuals belonging to 23 species was collected in the pitfall traps *(Table 1)*. Number of individuals and species have been found to be the highest in the non-managed stand, followed by that of the stand which was put under acorn after clearing the herbaceous and shrub layer. The fewest individuals and species were observed in the stand managed by grubbing and deep loosening *(Table 2)*. There was no significant difference (t-test, df>120, p>0.05) between the ES(380)-diversity (expected number of species calculated for 380 individuals) of the control stand and the stand put under acorn after clearing the herbs and shrubs, while both values were found to be significantly higher (t-test, df>120, p<0.05) than that of the habitat managed by grubbing and deep loosening *(Fig. 1)*. Composition of the carabid assemblage of the non-managed control stand and that of the stand cleared and put under acorn were similar to each other, while the carabid assemblage of the stand managed by grubbing and deep loosening was different from the assemblages of the two above stands *(Fig. 2)*.

Table 1

Number of individuals of the carabid species caught in the studied forest stands

	Non-managed stand	Stand managed by clearing the herbs and shrubs	Stand managed by grubbing and deep loosening
Carabus cancellatus	515	356	233
Carabus convexus	11	23	7
Carabus granulatus	37	2	1
Carabus violaceus	33	3	2
Notiophilus palustris	2	2	0
Bembidion lampros	0	0	1
Harpalus rufipes	2	23	2
Acupalpus meridianus	0	0	1
Poecilus cupreus	3	6	3
Pterostichus anthracinus	25	4	0
Pterostichus macer	0	1	0
Pterostichus melanarius	1	0	26
Pterostichus niger	2	0	0
Pterostichus oblongopunctatus	85	28	2
Pterostichus ovoideus	4	6	2
Molops piceus	2	0	0
Abax carinatus	34	2	41
Abax parallelus	42	2	73
Agonum moestum	22	2	0
Agonum obscurum	1	0	0
Platynus krynickii	7	3	0
Chlaenius nitidulus	9	2	0
Brachinus crepitans	0	0	1

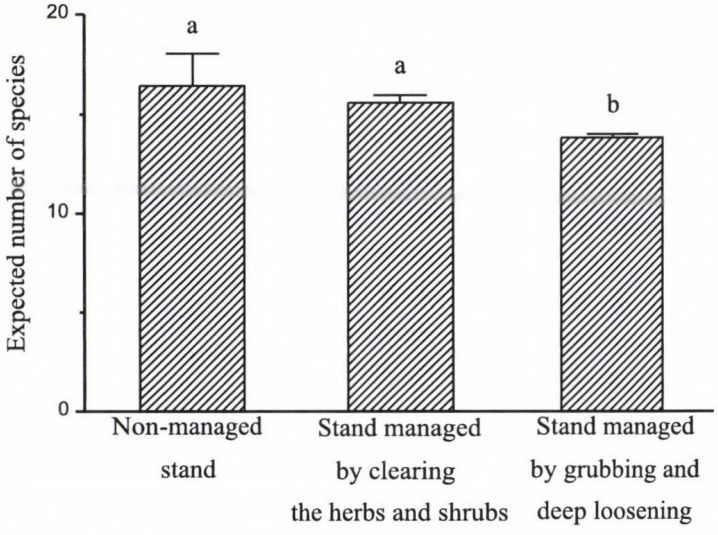


Fig. 1. Expected number of species calculated for 380 individuals (± S.D.) of the studied carabid assemblages. Different letters indicate a significant ($p<0.05$) difference by t-test

Table 2

Characteristics of the studied carabid assemblages

	Non-managed stand	Stand managed by clearing the herbs and shrubs	Stand managed by grubbing and deep loosening
Number of individuals	837	465	395
Number of observed species	19	16	14
ES(380)	16.4466	15.6057	13.8427

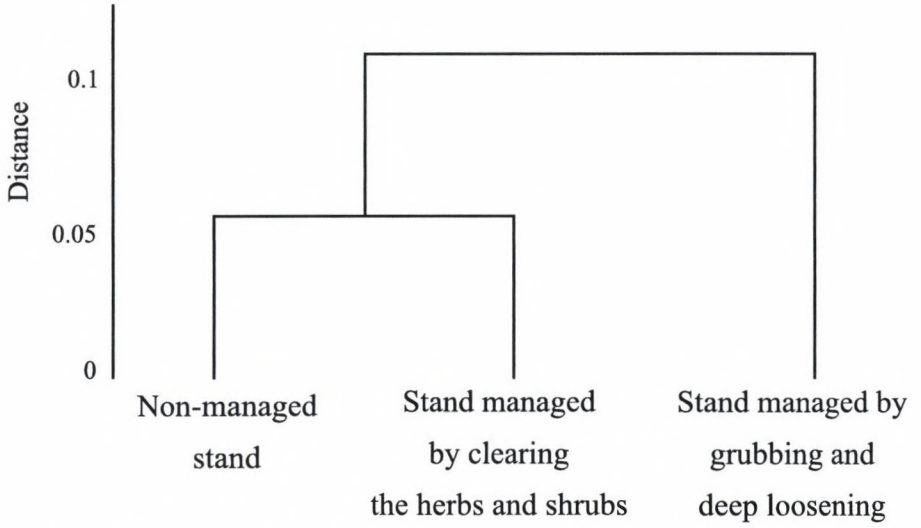


Fig. 2. Similarity of the studied carabid assemblages by cluster analysis. The Bray–Curtis index of similarity and the Ward–Orlóci fusion method was applied

Discussion

Effects of forestry practices on carabids

Our results proved that grubbing and deep loosening management practice completely changed the structure and composition of carabid assemblages. Our research also indicated that even many years (16 years) after the management there were significant differences in the species richness and the composition compared to the non-managed stand *(Figs 1* and *2)*. These changes can be explained with the fact that grubbing and deep loosening on the one hand promotes the extinction of carabid imagoes and larvae, on the other hand it homogenises the forests. Namely, during the grubbing and deep loosening practice the dead and decaying trees, the herbs, shrubs, and leaf litter are removed and structural heterogeneity of the soil (e.g. moisture, porosity etc.) is also disturbed. Previous studies demonstrated that the dead and decaying trees (Niemelä,

1997), the heterogeneity of the vegetation (Niemelä, 1990; Blake et al., 1996; Niemelä et al., 1996; Butterfield, 1997; Ings and Hartley, 1999), the deep leaf litter (Niemelä et al., 1992a, 1996; Eyre and Luff, 1994; Magura et al., 2000) and the structural heterogeneity of the soil (Luff et al., 1989, 1992; Rushton et al., 1991; Niemelä et al., 1992b; Baguette, 1993; Eyre and Luff, 1994; Šustek, 1994) were important factors determining structure and composition of carabid assemblages (Penev, 1996). The above-mentioned factors contribute to the development of microhabitats with suitable microclimate (air moisture, temperature, humidity) for carabids. Previous studies revealed that the greater heterogeneity of habitats (Niemelä et al., 1986, 1988, 1996; Báldi, 1990; Fuisz and Moskát, 1992; Blake et al., 1996; Magura and Tóthmérész, 1997, 1998; Magura et al., 1997) and the accompanying suitable microclimate (Niemelä et al., 1986, 1992b; Niemelä and Spence, 1994; Spence et al., 1996) increased the number of individuals and species richness of carabid assemblages. Moreover, the heterogeneity of habitats and the favourable microclimate may also increase the abundance and species richness of the decomposer and herbivore invertebrates that may serve as potential preys for carabids. The abundant preys may also influence the number of individuals, the species richness and composition of carabid assemblages (Niemelä et al., 1986, 1988, 1992a; Niemelä, 1990, 1993b; Halme and Niemelä, 1993; Lövei and Sunderland, 1996; Butterfield, 1997; Guillemain et al., 1997). Furthermore, the abundant and in time evenly distributed food resources may decrease the intraspecific (Loreau, 1990), interspecific competition among carabids (Lenski, 1982, 1984; Loreau, 1989) and the intraguild predation (Currie et al., 1996), which also play a crucial role in the organisation of carabid assemblages (Niemelä, 1993a).

Our results showed that management by clearing the herbaceous and shrub layer followed by putting under acorn also had effects on the carabid assemblages. The number of trapped individuals and species was fewer in this stand than in the non-managed stand *(Table 2)*. Compared to the non-managed stand the number of individuals of *Carabus granulatus* Linnaeus, 1758, *Carabus violaceus* Linnaeus, 1758 and *Pterostichus oblongopunctatus* (Fabricius, 1787) decreased significantly in the stand managed by clearing the herbs and shrubs. Moreover, *Pterostichus niger* (Schaller, 1783) and *Molops piceus* (Panzer, 1793) were absent in the stand that was managed by clearing of the herbs and shrubs *(Table 1)*. The expected number of species (ES(380)-diversity) has been also found to be fewer in the stand that was put under acorn after clearing the herbs and shrubs than in the non-managed stand, although the difference in the ES(380)-diversity was not significant *(Fig. 1)*. These changes can be also explained with the above discussed direct (habitat heterogeneity, microclimate) and indirect (food resources, intra- and interspecific competition and intraguild predation) effects deriving from the clearing of the herbs and shrubs. However, the result of the cluster analysis proved that the composition of the carabid assemblages of the stand managed by clearing the herbs and shrubs did not change significantly and was similar to that of the non-managed control stand *(Fig. 2)*. Several hygrophilous carabid species [*Notiophilus palustris* (Duftschmid, 1812), *Pterostichus anthracinus* (Illiger, 1798), *Agonum moestum* (Duftschmid, 1812), *Platynus krynickii* (Sperk, 1835) and *Chlaenius nitidulus* (Schrank, 1781)] were present only in the non-managed stand and in the stand managed by the clearing of herbs and shrubs which

indicates a similar soil moisture condition. These species were absent in the stand managed by grubbing and deep loosening reflecting the changes in the soil conditions caused by the forestry practice.

Implication for nature management

The establishment of non-native plantations destroys the habitats of the native forest carabids, thus directly endangering their existence (Szyszko, 1986; Desender and Turin, 1989; Magura et al., 1997). Moreover, the expansion of plantations and the reduction of native forests have an indirect effect: the native forest assemblages are also damaged as a result of the fragmentation (Den Boer, 1990; Niemelä et al., 1993; Spence et al., 1996; Niemelä, 1997). Because of these facts the management and renewal of the forests which are composed of native deciduous trees came into limelight. In Hungary 90% of the forests were renewed by clear cutting (Mátyás, 1996). However, the clear cutting changed the native forest assemblages drastically (Mader, 1986; Šustek, 1984; Niemelä et al., 1994), therefore a demand arose for the elaboration of an ecologically sound forestry practice (Niemelä, 1997).

Our results showed that forestry practice by grubbing and deep loosening completely changed the structure and composition of carabid assemblages and these changes can be observed even many years after the management *(Figs. 1, 2, Table 2).* Therefore, this forestry practice is not recommended to be used for forest management. Management by putting under acorn after clearing the herbs and shrubs did not substantially change the structure and composition of the native carabid assemblages *(Figs. 1* and *2, Table 2),* so it can be used for forestry management. However, during the application of this forest management practice the felling of the aged trees is unadvisable before the closing of the canopy layer of the recovering seedlings. Selective logging is to be recommended to promote the growing and closing of the renewing seedlings. Because of the felling of the aged trees before the closing of the canopy layer of the recovering seedlings, the light regimes and the microclimatic conditions may change considerably due to the decreasing canopy closure. These changes endanger the survival of the native forest specialists and promote the colonization of the habitat generalists and carabids typical of open habitats (Niemelä, 1993b, 1997; Niemelä et al., 1988, 1993, 1994). In the present study it was also observable that even 16 years after the management habitat generalists and carabids characteristic of open habitats (e.g. *Bembidion lampros* (Herbst, 1784), *Acupalpus meridianus* (Linnaeus, 1767), *Pterostichus melanarius* (Illiger, 1798) and *Brachinus crepitans* (Linnaeus, 1758)) were present in the forest stand managed by grubbing and deep loosening *(Table 1).* These species could colonise in the forest stand when the seedlings were young and the canopy did not close. The selective logging is to be recommended to promote the growing and the closing of the canopy layer of the recovering seedlings, because it may better preserve the original forest conditions and may therefore have moderate effects on the original forest fauna and flora (Atlegrim et al., 1997).

Our results also suggest that the sensitivity of carabids to environmental variation and their rapid responses to habitat changes (Eyre and Rushton, 1989; Eyre et al., 1990; Desender et al., 1994; Blake et al., 1996) make them a suitable indicator group to

predict and assess the effects of forest management practices. However, for the elaboration of an universally applicable ecologically sound forestry practice we must possess efficient knowledge of the assemblages of both the different trophic levels and the various geographic locations.

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First Report of *Dactylispa lenta* Weise (Coleoptera: Chrysomelidae) as a Vector of Rice Yellow Mottle Virus

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Rice yellow mottle virus (RYMV) is a severe disease of rice in Africa. It is naturally transmitted by several species of beetles. *Chaetocnema pulla* is widely believed to be an important vector of RYMV in Tanzania. However, the high incidence of RYMV in some rice fields where *C. pulla* was not seen in large numbers indicated that more vectors of the virus might exist. Transmission studies conducted showed *Dactylispa lenta* to be a vector of RYMV in Tanzania. This has not been previously known.

Keywords: *Chaetocnema pulla, Dactylispa lenta,* rice yellow mottle virus.

Tanzania is the largest producer and consumer of rice in the East, Central and Southern Africa (ECSA) region after Madagascar. Rice is grown in different ecologies (upland, lowland and irrigated) and the major producing regions are Mbeya, Morogoro, Mwanza and Shinyanga.

Rice yellow mottle virus is specific to Africa and it is concentrated in the eastern and western parts of the continent. It is clearly the most important disease of rice in Tanzania. It has spread fast and now is found in almost all the rice growing regions.

In countries where the disease is prevalent, *Chaetocnema* spp, *Dicladispa* spp, *Trichispa sericea* and *Sessilia pussila* are reported to be important RYMV vectors and the disease is also mechanically transmissible (Bakker, 1974; Reckhaus and Andriamasintseheno, 1997; Abo, 1998). Out of the above-mentioned vectors only *Chaetocnema pulla* was encountered in this study. It was observed that in fields where *Dactylispa lenta* was found and *Chaetocnema pulla* was not seen in large numbers, the RYMV incidence was usually higher than in fields where *Dactylispa lenta* was absent. This gave the indication of *Dactylispa lenta* as a possible vector of RYMV and hence necessitated this study.

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Materials and Methods

The experiment was conducted in the screenhouse. The mean temperature in the screenhouse was 23 °C (maximum 31 °C and minimum 16 °C) during the study. Seeds of variety 'Supa' which is susceptible to the disease were used. The insects *(Dactylispa lenta)* were collected from rice fields in Morogoro and Mbeya regions which are widely known to be 'hotspot' regions for this disease in Tanzania. The insects were collected by the use of aspirators and sweepnet. During transportation from the field, the insects were kept in containers (bottles) closed by a perforated cover with a closable opening. An electronically operated aspirator and camel hairbrush were used to collect and transfer insects from plant to plant and from cage to plant. Wooden cages measuring 70 cm x 80 cm x 70 cm were used in keeping the insects before being used for the transmission tests. Another improvised cage as used by Banwo and Harrington (1998) was used to retain insects on virus source plants (acquisition feeding). Cellulose acetate tubes measuring 2.5 cm with a netting top were used to retain viruliferous insects on test plants (inoculation feeding). Acquisition feeding was for 3 days on potted rice plants inoculated about three weeks earlier. Also, inoculation feeding was for 3 days on potted young rice seedlings at the 2 to 3 leaf stage, transplanted 2 days earlier. Rice plants are more susceptible to the disease at the seedling stage (Bakker, 1974). Adult insects were used in the transmission tests since they are responsible for the dissemination of vectored viruses under most field conditions (Fulton et al., 1987). One insect per test plant was used. The plants were kept in the screenhouse for three weeks for symptom observation. The Enzyme Linked Immunosorbent Assay (ELISA) test as described by Clark and Adams (1977) was conducted in the laboratory to determine which plants were infected. Ten potted plants were used for each of the different vector populations (1, 2, 3 and 4) and the experiment was run/replicated 5 times. The transmission percentage for each of the vector populations was then obtained.

Results and Discussion

The results of the transmission tests *(Fig. 1)* clearly show that *Dactylispa lenta* was able to transmit RYMV to healthy seedlings. The insect caused similar foliage damage typical of beetles on the rice plants. Interestingly however, earlier reports (e.g. Nye, 1960: De-Pury, 1975 and Akhabuhaya and Lodenius, 1988) did not show the presence of *Dactylispa lenta* on rice in Tanzania. This indicates that the number of important insect species on rice might have increased over the years. Bakker (1974) reported *Dactylispa bayoni* as a vector of RYMV in Kenya. However, this is the first report of *Dactylispa lenta* as a vector of RYMV. It confirms reports that pest problems may vary from one area to another in view of the diverse conditions under which rice is grown (Grist and Lever, 1969; Alam, 1984; Heinrichs, 1994).

Unlike *Chaetocnema pulla, Dactylispa lenta* was not found in all the fields visited hence might be considered to only be potentially an important vector of RYMV in Tanzania.

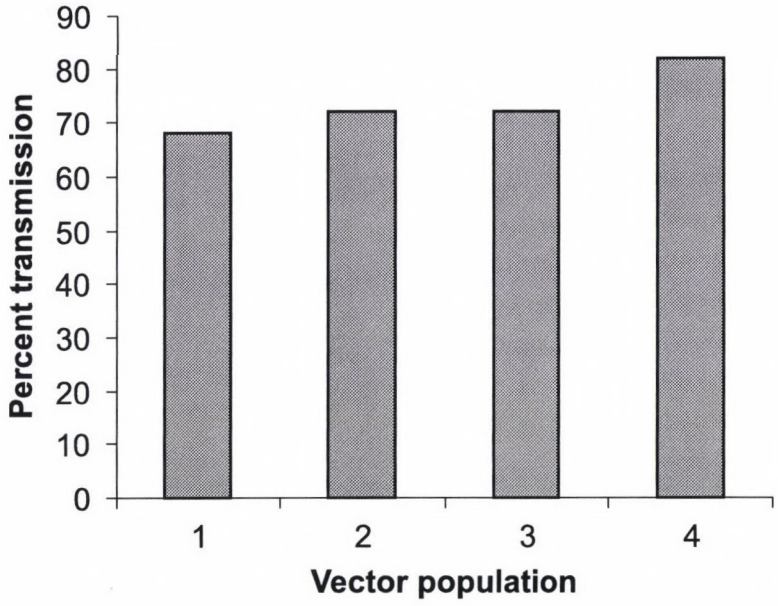


Fig. 1. Transmission of RYMV by *Dactylispa lenta* (Coleoptera: Chrysomelidae)

Acknowledgements

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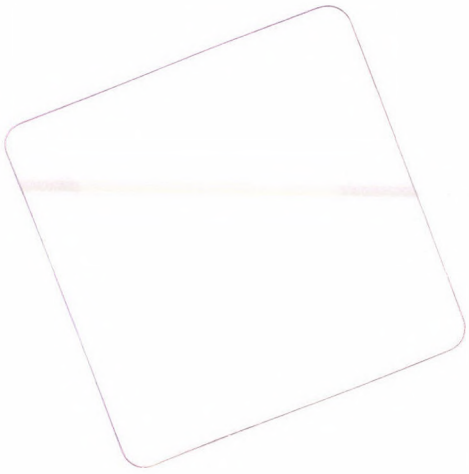
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New Data to the Knowledge of *Calepitrimerus vitis* Nalepa in the Vine-growing Region of Szekszárd, Hungary (Acari: Eriophyidae)

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The eriophyid mite *Calepitrimerus vitis* is a common pest in many vineyards in Hungary. Deutogynes (winter-form) of *Calepitrimerus vitis* in the vineyards of Szekszárd emerge from their overwintering sites in spring. Protogynes (summer-form) start to appear at the beginning of May, replacing deutogynes gradually. The process lasts until the end of May when the deutogynes disappear. The mite population increases slightly during the summer months. In August a rapid increase occurs. The maximum density of mites is reached at the middle of August or at the beginning of September, when the first deutogynes appear. The mite density declines in September rapidly and it is very low in October.

The hibernation shelters are in the buds and at the cane base with the 2 year old wood. The most females are found at the cane base and in buds 1–4. In upper buds mite density gradually declines. A washing technique is described to estimate the population of *Calepitrimerus vitis* and useful for sampling of eriophyid mites either in summer or winter.

Keywords: *Calepitrimerus vitis*, eriophyid mite.

The eriophyid mite *Calepitrimerus vitis* Nalepa was detected in vineyards of Szekszárd first in 1979 and it has been causing significant damage since 1982. The control of this pest is not effective and so it is important to collect information on its biology and ecology. In case of mite infestation leaves curl and growth of the shoots and leaves is retarded. High level of infestation in spring may cause as much as an 85% decrease in the weight of the grapes at harvest time (Pérez-Marín, 1992). Population dynamics and hibernating shelters of *Calepitrimerus vitis* Nalepa have been studied in several other countries (Carmona, 1978; Baillod and Guignard, 1986; Liguori, 1987, 1988). However, investigation is required for each region for the development of integrated and biological pest management programmes, and so it is necessary to collect information on the biology and ecology of this pest in the vineyards of Szekszárd. A washing technique is described to estimate the population of *Calepitrimerus vitis,* useful for sampling of eriophyid mites either in summer or winter.

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Materials and Methods

For the study of the population dynamics of *Calepitrimerus vitis* samples were taken in the period April–October in 1997–1999. The trial was carried out in Chardonnay grape variety, the plot size was 0.2 ha. No insecticide treatment was carried out in the vineyards for the past 5 years.

Sampling started in the spring of 1997, after bud burst. Samples were taken in the spring every 3 weeks, in the summer every 4 weeks. A sample of 30 leaves was collected in each time, chosen the leaves from the plot randomly. The leaves were chosen from the apical shoot area since these leaves had a high density of mites.

A shaking technique was carried out in water to move mites from the leaves. The solution was tinted and after sieving the mites were counted on the sieving-disc under binocular microscope at 50 x magnification.

Leaves were broken into small pieces in a 300 ml glass pouring out 150 ml tap water. An LE-203 type shaking machine was applied for a half an hour to move mites form the leaf surface to the solution. This technique substitutes the solving material and the individually handy washing. It is important because the solving material in many cases kills the mites. Our shaking technique let us observe living mites under binocular microscope. After shaking the solution was tinted with "Azúr II. eozin" microscopical colour. The tinted solution was sieved through a two-phase vacuum-sieving. The size of first sieving-tissue is 300 μ, the second is 40 μ, the latter is a changeable sieving disc. The two sieving surface occupy a "one under another" position in a plastic sieving-box fitting in a vacuum-tube. In the first sieving phase the rough dirt and leaf pieces are separated, in the second phase the mites are caught.

The blue background is optimal for observing mites under microscope. The changeable sieving disc is 3 cm in diameter. The mites were observed under binocular microscope at 50 x magnification. The observing unit was a microscopical visual field. A microscopical visual field at the 50 x magnification is 0.2 cm^2 (0.196 cm^2). The area of sieving disc is 7 cm^2 (7.065 cm^2), that is 35 microscopical visual fields. The number of eriophyids per leaf was calculated from the average number of mites found on sieving disc.

Investigation of deutogyne distribution in cane was carried out with a bud washing technique. In accordance with the observation of Schruft (1966), Sántha (1981) and Petter (1984) the hibernation shelters of deutogynes are the buds and the two year old woody bark. The samples were taken on 2 December 1997 and on 1 December 1998. 30 vine canes – picked at random – were collected. The canes were collected with the two year old wood. As the buds are often infested differently, pairs of successive buds were made. Thus six pairs of buds were investigated (1–2, 3–4, 5–6 ... 11–12) in each cane. The cane base and the 2 cm long two year old wood gave the sample number 7. The number of mites collected from buds in the same position gave the basis to calculate the average number of deutogynes in bud pairs.

The buds were examined with washing-out technique. The procedure was the same as mentioned above. The buds were gently dissected with a scalpel in a 300 ml glass pouring out 100 ml tap water. After shaking and colouring the samples were sieved. The

mites caught on the sieving disc were observed then under binocular microscope. Their number was calculated as mentioned above.

Results and Discussion

Population dynamics of Calepitrimerus vitis *Nalepa in the vineyards of Szekszárd*

During investigation of the samples in the 3 experimental years we got information about the population dynamics of this pest in our region. The results are shown in *Fig. 1.* No insecticide treatments were carried out in the vineyard for 5 years which might directly have an acaricidal effect.

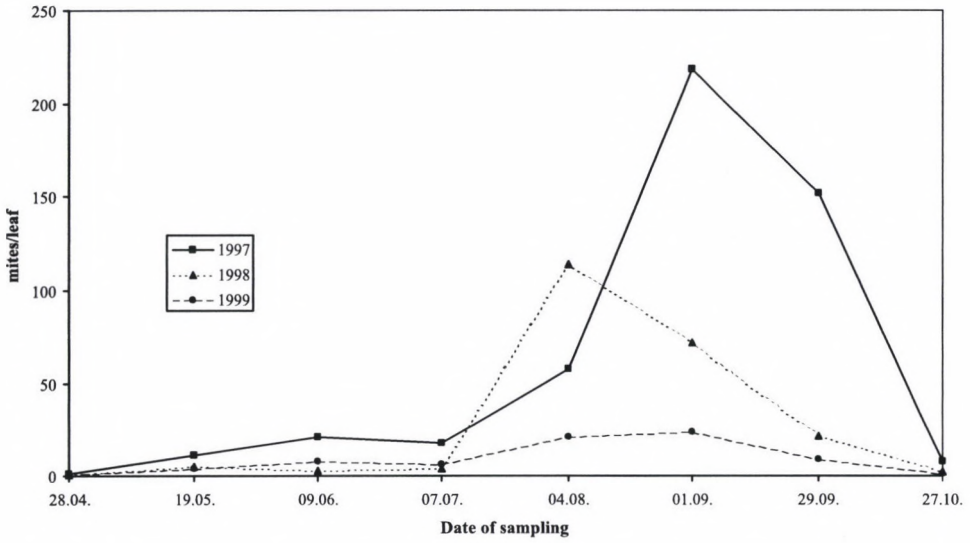


Fig. 1. The population dynamics of *Calepitrimerus vitis* Nalepa in 1997–1999

Population dynamics in 1997 (Fig. 1)

The mite density was low in the spring in 1997 (1 mite/leaf), and remained low with a slight increase until the beginning of July. A rapid increase occurred from the beginning of August. The maximum density (218 mites/leaf) was reached at the end of August and in the first days of September. During September the mite density remained high and we observed the pest both on the surface and the back side of leaves. The mite density decreased rapidly from the end of September and reached a low value upto the end of October.

The warm, dry summer was favourable to the mass multiplication of *Calepitrimerus vitis*. In 1997 the high temperature and the low precipitation resulted in high level of mite infestation. The temperature and precipitation were average in June and July. From the beginning of August to the end of October a warm and dry period succeeded and this period resulted in the increase of mite density. The mean temperature in August was higher than the former two months and the precipitation was low. The September was also dry and warm. Although the mite density rapidly decreased from the beginning of October, a portion of mites still stayed on the leaves at the end of the month. The mite density in the sample collected at the end of October was still 8 mites/leaf.

Population dynamics in 1998 (Fig. 1)

In the spring of 1998 the mite density (0.5 mite/leaf) was lower than in the spring of 1997. The number of mites was low until the beginning of July as also observed in 1997. The mite density increased rapidly from the beginning of July and reached the maximum value (114 mites/leaf) at the beginning of August. After that the mite density gradually decreased to the low level to the end of September. In 1998 the infestation level was lower than in 1997, the maximum density was reached earlier and taking refuge was started earlier. The reason for the low mite density in spring may be the high level of precipitation in May and June. The mean temperature during the summer months was more favourable for mites than in the former year. In 1998 the permanent precipitation of the summer and the autumn months and the high level of relative humidity had an unfavourable effect on the mite population and blocked the multiplication of *Calepitrimerus vitis*.

Population dynamics in 1999 (Fig. 1)

In the spring of 1999 the mite density (0.5 mite/leaf) was low. The population remained low during the whole year. Only in August and September could be observed a slightly increase in the mite density. The maximum density (24 mites/leaf) was reached at the beginning of September. This value was much more lower than in the former years. In the second half of September the mite density decreased and remained low until the end of October. The reason for the low mite density in 1999 was definitely the weather conditions. The average temperature in the summer and the autumn months appeared to be favourable to the multiplication of mites. However the precipitation 405 mm (June 157 mm, July 180 mm, August 68 mm) falling during the summer months and the high level of relative humidity blocked the multiplication of mites. The September and October were warm and dry but taking the previous interval into consideration in spite of the favourable weather conditions there was no increase in mite density during the autumn months. The dry and warm summers appeared to favour high population of mites as noted by other researchers (Baillod and Guignard, 1986; Pérez-Moreno and Moraza-Zorrilla, 1998). The warm and dry summer of 1997 produced higher density than in the former two years. In these years (1998, 1999) the average temperature was similar, but the precipitation was much more than in 1997 *(Figs 2, 3)*.

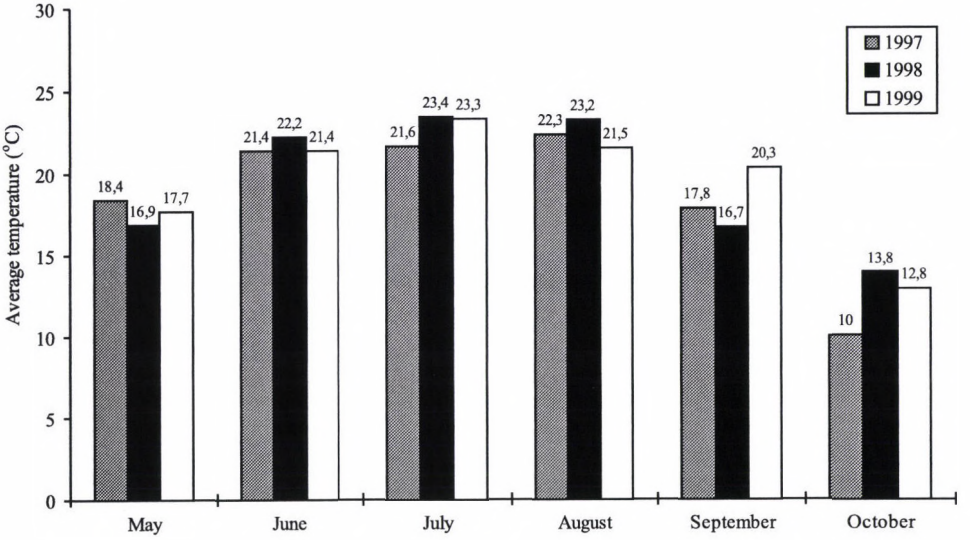


Fig. 2. The mean temperatures from May to October during 1997–1999

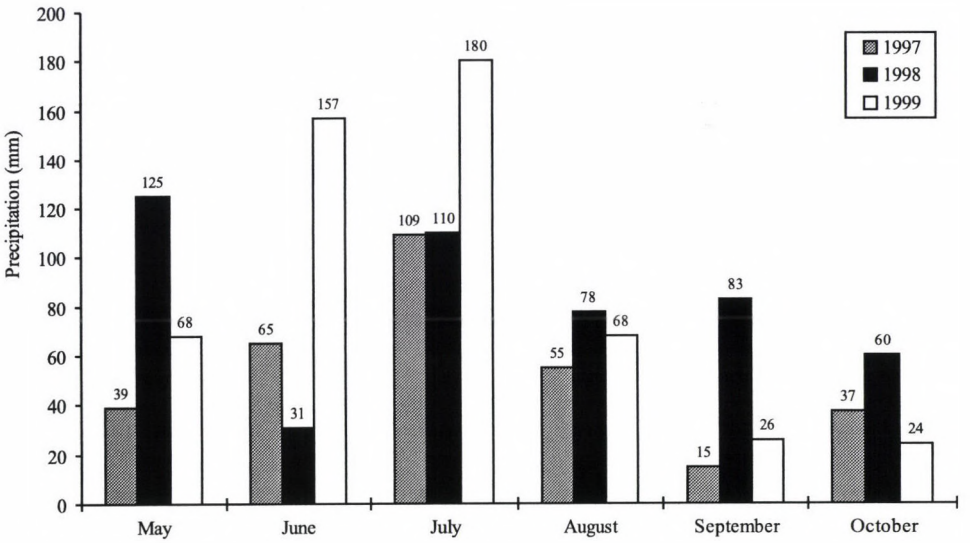


Fig. 3. The mean temperatures from May to October during 1997–1999

General dynamics

The deutogynes leaves their hibernating shelters in spring before immediately bud burst and at the bud burst. The pest density is low at this time. The first summer forms (protogyne) appear in the first half of May and protogynes relieve deutogynes until the end of May completely. The mite population increases slightly until the beginning of July and from this time a rapid increases occurs. The maximum density is reached from the beginning of August to the middle of September. At this time, in accordance with the observations of Carmona (1978) the first deutogynes are detected. At this time the deutogynes start to move towards the hibernating shelters and so the mite damage on leaves rapidly decreases during September and is very low in October. The first appearance of deutogynes in summer appears to depend not only on the environmental conditions such as morphological and physiological modifications of the foliage (Kreiter and Planas, 1987) but also depends on the number of mites. In those years when the mite density is high, deutogynes appear sooner than when the population levels are lower. It was observed by Carmona (1978) and he attributed it to a reduction of the foliage in the heavily infested vineyards. A large number of mites feeding on the same leaf accelerates the process of tissue hardening. This makes mite feeding difficult and is responsible for deutogyne development in eriophyid mites, as noted by Keifer (1975).

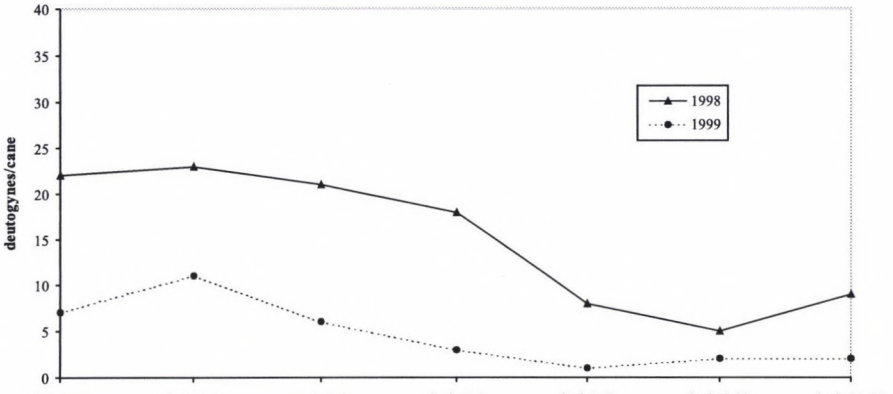
The results of the three years show that the high temperature, the low level of precipitation and the low relative humidity have a large influence on *Calepitrimerus vitis* population. The warm, dry summers result in an increase of mite population.

Distribution of hibernating females in the buds of canes

The results of bud washing obtained in winters of 1998–1999 for deutogynes distribution on the cane are shown in *Fig. 4*. The first deutogynes appear in August and they start to move towards the hibernating shelters.

In *Fig. 4* it can be seen that the distribution of mites within the cane is the same in 1998 and 1999, but there is a difference in the number of hibernating mites in 1998 and 1999. The cane base with the two year old wood and the first two bud pairs (buds 1–4) tend to shelter higher number of mites than the others. The most females are found at the cane base and in buds 1–4. But it is interesting to think about pruning. If we apply a "short pruning" procedure on the canes and remain only first 2 buds on every cane in the vineyards, we achieve a great reduction in the number of mites as the upper buds (buds 3–12) contain 45–57% of hibernating females. Consequently the short pruning is suitable for reducing the number of deutogynes. But in case of "long pruning" the canes are remained as long as of 10–12 buds and so the number of hibernating mites remains high which may cause spring damage.

If these results are compared to those from other countries the following can be found. In Portugal there are similar results to ours, the first buds tend to shelter higher number of mites (Carmona, 1978). In Switzerland and Spain the central buds (5–6, 7–8, 9–10) tend to shelter higher numbers of mites (Baillod and Guignard, 1986; Pérez-Moreno and Moraza-Zorrilla, 1998). In France high numbers of mites were found in the buds and



1998	21%	22%	20%	17%	7%	5%	8%
1999	22%	34%	19%	10%	3%	6%	6%

Fig. 4. The mean number of deutogynes at the cane base and in the buds in 1998–1999

reduced population were found under the bark at the cane base (Gay-Bellile, 1977). In Germany in accordance with the observation of Schruft (1966) deutogynes were exclusively located in the buds.

In Hungary Sántha (1981) and Petter (1984) publish information on the distribution of hibernating females within the cane. According to the former author small portion of mites hibernates in buds and great portion of mites hibernates at the cane base and the two year old woody bark. 100% of hibernating females were found at the cane base and in the first 5 buds. There was no found mites in the upper buds. According to the latter author the mite population is located at the cane base and in the first 5 buds, exception of Sylvoz cultivation system, where without exception the hibernating mites appear again in buds 7–10.

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Light-Trapping of the European Corn Borer (*Ostrinia nubilalis* Hbn.) at Different Values of the Q-index Expressing the Different Intensities of Solar Flares

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The paper deals with connections between solar flare activities and light-trap collection of insects. The authors have worked out the catch data of European corn borer (*Ostrinia nubilalis* Hbn.) adults, as published for the period of 1976–1997 by the Hungarian national light-trap network. The results proved that both the daily and annual catches were significantly modified by the Q-indexes, expressing the different lengths and intensities of the solar flares. On days with high Q-indexes relative to the ones of the average swarming periods, the number of catches are considerably lower. In those years when the Q-index is high, the average individual number and the number of caught moths are lower by 30% as compared to the average number of total cycles (11 years) and the average population density of a given biotope. On the contrary, in years with low Q-indexes an increase as high as 45% can be experienced in the number of individuals collected. Thus, by evaluating the light-trap catches a strong modifying effect of solar flares has to be considered.

Keywords: European corn borer, *Ostrinia nubilalis,* light-trap, solar flares, Q-index.

As part of global solar activity flares, eruptions can be observed in the active regions of the solar surface, that are accompanied by intensive X-ray, gamma- and corpuscular radiations, that reach also the Earth and establish an interaction with its outermost atmosphere, producing thus changes in its electromagnetic conditions (Smith and Smith, 1963).

The Sun eruptions, flares are short (lasting max. 10–20 minutes) shiny spots, temporal areas in the chromosphere of the Sun. Their observation is carried out conventionally in the red (656.3 nanometer wavelength light) of alpha line of hydrogen. At the time of intensive flares the corpuscular emission is thousandfold as compared to a quiet period of Sun. The corpuscles are mostly electrons flying into all directions (also toward the Earth) with a speed of 1500 km^{-1}. These electrically charged corpuscles compose the so-called solar wind that – in contrast to the electromagnetic radiation that reaches the Earth in 8 and a half minutes – reaches our atmosphere in 26–28 hours. On their way to Earth, the flare particles have also to pass the interplanetary space. The magnetic field generated by the galactic cosmic radiation is moderating considerably the effects of flares exerted on the magnetosphere of Earth. Thus, not all flares cause changes in the physical conditions of the upper atmosphere. If, however these changes result from the above-mentioned reasons, the weather becomes temporarily altered and the magnetic field of the corpuscles modifies the undisturbed daily course of the terrestrial magnetic field.

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The flares are classified according to the size of their area as compared to the total solar surface. The flares of primary importance (1) do not reach 250 times the half of on millionth part of the total solar surface. If the flare takes 250–600 times this size, it receives an index number of 2; if greater 600 times than that, it has a significance of 3. Because of their significant energy emissions, the cosmic influence of the flares No. 2 and 3 is the most considerable.

Most daily flare activities are characterised by most authors by index Q that expresses the significance of flares also by their duration. Its calculation is made by the following formula:

$$Q = (i \times t)$$

where i = flare intensity, t = the time length of its existence

Earlier Örményi (1966) calculated and published the flare activity numbers based on similar theoretical principles ("Flare Activity Numbers") for the period of 1957–1965.

The solar activity also exerts influence on life phenomena. In the literature accessible to the authors, however, no publication can be found that would have dealt with the influence of flares on the collection of insects by light-traps. Earlier we have published our studies and demonstrated the influence of hydrogen alpha flares No. 2 and 3 (Tóth and Nowinszky, 1983) on light-trap catches.

Material

The Q-index daily data for the period 1976-1997 were provided by Dr. T. Atac (Bogazici University, Kandilli Observatory and Earthquake Research Institute, Istanbul) and by Dr. J. Verő (Research Institute of Geodesy and Geophysics, Hungarian Academy of Sciences, Sopron). Their help is here gratefully acknowledged.

The collection data of European corn borer (*Ostrinia nubilalis* Hbn.) were taken from the collection of the national light-trap network. The data of traps, operated by the Plant Protection Stations were used with the authorization of Dr. I. Eke (Ministry of Agriculture and Country Development), Dr. M. Tóth and Mrs. Gy. Mohai (Station of Plant Health and Soil Protection, Budapest). Many data were received from Professor Z. Mészáros (University of Horticulture and Food Industry, Budapest).

In course of the period studied: in 81 light-trap stations and 3114 nights 133 419 moth individuals were collected in total. As more than one trap operated on each nights the authors disposed over 40 336 observation data in preparing present paper.

Methods

From the collection data of the European corn borer (*Ostrinia nubilalis* Hbn.) relative catch (RC) data were calculated for each observation posts and days. The RC is the quotient of the number of individuals caught during a sampling time unit (1 night or 1 hour) per the average number of individuals of the same generation falling to the same

time unit. In case of the expected average individual number the RC value is 1. The introduction of RC enables us to carry out a joint evaluation of materials collected in different years and at different points.

At the values of Q-index showed considerable differences in course of the respective years, they were preferably expressed as percentages of the averages of swarming periods. In the first step we studied the influence of flare activities on the daily catches. To disclose the latter, the Q/Q average values were co-ordinated with the relative catch data of different observation posts for each day of the catch period. The Q/Q mean values have been contracted into groups (classes), then averaged within the classes the relative catches data pertaining to them.

In subsequent studies we have investigated, whether the constantly changing activities of flares had modified the numbers of trapped European corn borer (*Ostrinia nubilalis* Hbn.) individuals? For this study only the data of those observation posts could be used that had worked throughout at least one total solar activity cycle (1976–1986 or 1986–1997). The light-traps operated through both cycles only on two observation posts; additionally, in the first cycle 11 and in the second further 1 light-trap yielded data from a total period. The total catch of the individual years has been compared with the average catch number of 11 years, so essentially yearly relative catch data (RCy) were calculated. These numbers were yearly compared to the average of Q-index, then in years with low (Q-index average 1–3), medium (Q-index average 3–10) and high (Q-index average higher than 10) flare activity the level of significance of relative catch data (RCy) deviation was tried by t-test.

Results

The connections between Q/Q averages and daily catches of European corn borer (*Ostrinia nubilalis* Hbn.) are presented in *Table 1.* The values of Q-index characteristic for each year are shown in *Table 2.* In the latter the yearly relative catch data (RCy) of the studied species are also presented.

Discussion

From the results several important consequences could be drawn. Between the Q-indexes and maximal values of Q/Q average a negative correlation ($r = -0.715$) could be found, that is significant at 99.9 % level.

As proven by the data of *Table 1,* on those days when the value of Q/Q average surpasses 3, the catch number of European corn borer (*Ostrinia nubilalis* Hbn.) adults significantly regresses. Such high values occur mainly in years in which the Q-index averages are lower than 10. This means that in those years when the flare activity is mostly moderate the rarely occurring high Q-indexes surpass considerably the average. In those years, however, where the average Q-index is higher than 10, the highest Q-index

Table 1

Daily catches of European corn borer (*Ostrinia nubilalis* Hbn.) adults in 1976–1997 at different values of Q/Q average

Q/Q average 1	Average of relative catch 2	Number of data 3
0.00	0.964	9541
0.12	1.051	3144
0.31	1.054	3626
0.51	0.988	4112
0.71	1.048	3673
0.91	0.985	2695
1.43	1.008	8488
2.41	0.987	2666
3.42	1.102	911
4.97	0.870	825
6.82	0.821	321
12.21	0.783	334

Notes: Correlation between Q/Q average and the relative catch:
r = –0,8146 (significant on levels higher than 99%)

values surpass much less the average. The unfavourable influence of flare activity, which exceeds considerably the average, is reflected in the decreasing number of daily light-trap catches. Because decreasing catches may be experienced on the first day of strong flare activity, we assume that the success of light-trapping is significantly and negatively influenced by the electromagnetic radiation arriving from the Sun. The decrease of catch numbers cannot be felt at Q/Q average values lower than 4. To these values generally high Q-indexes are attached, at the same time the deviation from the average is lower.

One can suppose that the high flare activity generally decreases the number of caught individuals. The method used by us so far, i.e. the relative daily catches, calculated separately for each year per swarming period of the Lepidopteran is inadequate. By introducing and using the relative yearly catch (RCy) we succeeded to confirm the fact that in the case of high flare activity when the Q-index average was higher than 10 (the average catch number calculated for a period of 11 years, a Sun cycle and characteristic for the given observation point, does barely amount to 70% of the expected individual number collected. In years with low flare activity, however, the catch number exceeds the average at least by 44%.

So, according to our results, changes in Sun flare activity considerably modify both the daily catch of European corn borer (*Ostrinia nubilalis* Hbn.) individuals and the number of individuals present. Both statements are important for plant protection prognostics.

Table 2

Yearly relative catch (RCy) of European corn borer (*Ostrinia nubilalis* Hbn.) adults calculated as functions of Q-index yearly averages

Years	Q-index		Q/Q average	Number	RCy
	maximum	average	maximum	of data	average
1986	5.36	0.406	13.20	13	2.079
1996	11.48	0.478	24.02	3	1.052
1976	4.98	0.632	7.88	13	1.294
1997	12.28	0.642	19.13	3	0.443
1995	12.00	0.734	16.35	3	1.423
1994	12.08	0.973	12.42	3	0.734
1985	21.31	1.459	14.61	13	1.467
1987	16.21	2.917	5.56	3	1.289
a		*0.920*		*54*	*1.440*
1977	32.27	3.059	10.55	13	0.788
1983	37.29	3.360	4.03	13	1.163
1984	42.09	3.573	11.78	13	0.863
1988	36.90	6.880	4.37	3	1.280
1992	33.56	8.453	4.88	3	1.642
1993	36.92	9.243	10.99	3	0.634
b		*5.468*		*48*	*0.984*
1978	65.23	10.719	6.09	13	0.653
1979	56.40	12.151	3.46	13	0.536
1980	53.38	15.350	3.39	13	0.330
1981	51.29	15.757	3.12	13	0.737
1982	112.31	16.313	5.77	13	1.088
1989	55.10	16.461	3.33	3	0.766
1990	43.40	16.556	3.57	3	0.620
1991	68.22	19.464	4.44	3	1.115
c		*15.614*		*74*	*0.689*

Notes: Levels of significance:
Between Q-index average and maximum of Q/Q average 99.9%
Between *a–b* lines of RCy values 95.0%
Between *b–c* lines of RCy values 95.0%
Between *a–c* lines of RCy values 99.9%

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Investigation on the Colour Preference of *Thrips tabaci* Lindeman (Thysanoptera: Thripidae)

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Yellow proved to be the most reliable colour for monitoring of *Thrips tabaci*. White and blue could be used with weak, but still reasonable effectiveness.

Keywords: *Thrips tabaci*, coloured traps, monitoring *Thrips tabaci*.

Since *Thrips tabaci* has a significant role in the epidemic of tomato spotted wilt virus under Hungarian climatic conditions (Jenser et al., 1996) it was required to evaluate the monitoring of its flight period and activity. According to former investigations and experiences the coloured traps proved to be suitable for this purpose (Lewis, 1959; Beckham, 1969; Kirk, 1984; Yudin et al., 1987; Fernández and Lucena, 1990; Broadsgaard, 1993; Jenser, 1993). Because different colours (white, yellow, blue) were reported to attract *T. tabaci* (Czencz, 1987) we decided to conduct comparative experiment to determine its colour preference.

Materials and Methods

The experiments were done in two tobacco fields at Bököny (north-east Hungary) in August and Kiskunhalas (in the Danube-Tisza Interfluve) in September in 1999. The differently coloured (CSALOMON) traps (white, yellow, blue and greenish-yellow) were hung on a trap-rack four on each. The sequence of the colours was systematically varied, five times. The trap-racks were placed out 15 m from one another, at a height of 180 cm, at the top level of the plants. Both sides of the traps were coloured and covered by sticky material. The size of trap was 10 x 16 cm (320 cm^2). After 6 days of exposition the caught *T. tabaci* specimens were counted by Wild dissecting microscope.

The trap colours were measured by the Hunterlab Ultrascan spectro-colorimeter at the Szent István University, Department of Physics-Control, Budapest. The instrument was set in reflectance mode as described in followings.

Small area of view – round opening with a diameter of 1/4 inch (6.2 mm). Mounted without UV light source. Total reflectance, specular included (mirroring effect). Calibrated using the standard method: light trap, white and grey officially calibrated standards.

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The instrument built using the O/d observation standard: diffuse illumination, and observing (measuring) in 0 degree direction (perpendicular to the plane of the sample). The original observation standard was modified by Hunterlab using the 8 degrees standard. This method is developed for excluding the specular part of the light flux *(Fig. 1)*.

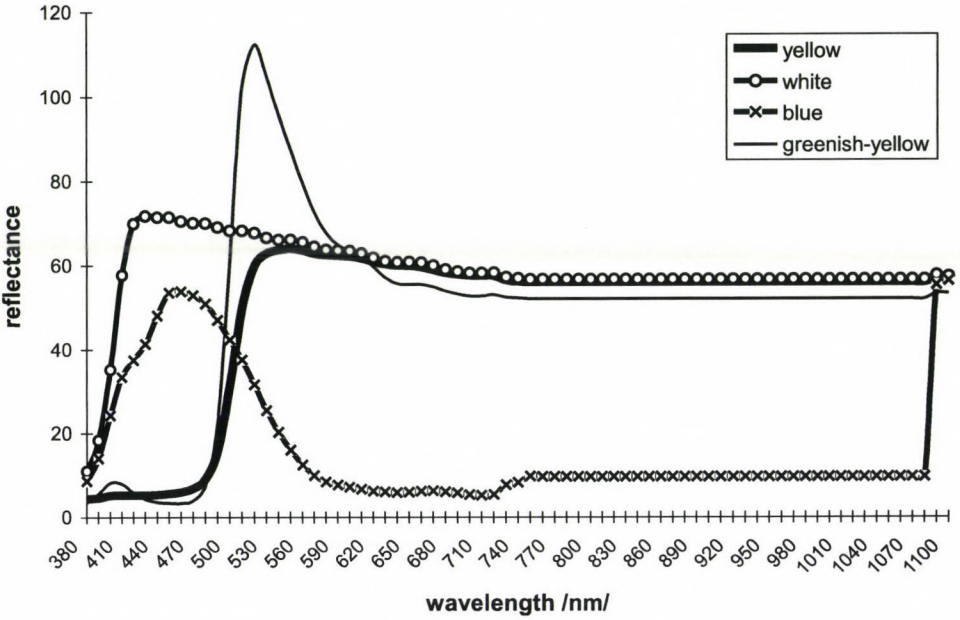


Fig. 1. Spectrum of the colours of the traps

The acquired data was stored as spectra from 380 to 1100 nm wavelength in 10 nm steps. The results were calculated by the original software belonging to the instrument in CIE 1931 XYZ system and in the CIE 1976 CIELAB system, computed according the 2 degrees standard observer the C standard illuminant (correlated colour temperature of 6774 K).

More colorimetry data were calculated by a locally developed software and this way the COLOROID type colour numbers were also computed. (This system has three basic data: A for the hue, T for the saturation and V for the lightness).

The statistical procedure was performed by Ministat 2.4 as follows: total caches of the different colour sticky sheets placed near each other were used for the comparison as related samples. After testing the hypothesis of stochastic homogeneity by Friedman test and ANOVA on ranks. Tukey-type pairwise comparison of means was performed.

Results and Discussion

Traps caught altogether 477 *T. tabaci* specimens at Kiskunhalas and 333 at Bökény. More than 36% of the specimens was attracted by the yellow traps and the rest of them distributed among the white, blue and greenish-yellow colours. Significant difference was established in the number of captured specimens between the yellow and the other colours in Bökény. Although yellow traps caught the specimens in high number, significant differences were not established in the attractiveness between yellow and blue colours in Kiskunhalas. The white and blue attracted the specimens in varying to a certain degree. No significant differences were established between the number of the attractiveness of the two colours. Greenish-yellow traps were the least effective *(Figs. 2, 3).*

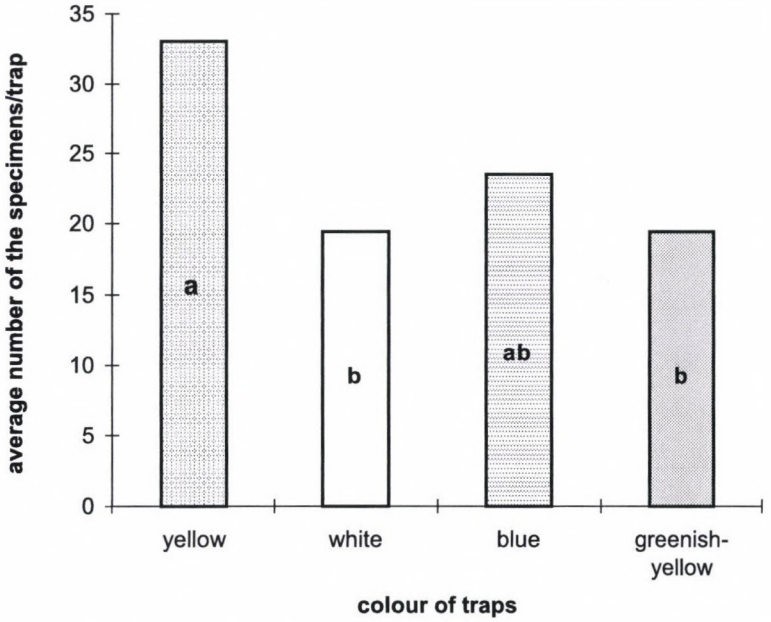


Fig. 2. Average number of *Thrips tabaci* specimens caught by the colour traps (Kiskunhalas, 1999) (Columns denoted by different letters are significantly different at a significant level $p < 0.05$)

Regarding the attractiveness of colours to *T. tabaci* is to some extent different. According to the data of Kirk (1984) as well as Yudin and co-workers (1987) white is the most attractive colour. On the other hand yellow and white (Fernández and Lucena, 1990), yellow and blue (Kirk, 1984; Broadsgaard, 1993), yellow, white and blue (Czencz, 1987) attract the specimens approximately with similar effectiveness. For the monitoring yellow (Bognár and Shanab, 1969), blue (Pénzes, 1994) and white (Jenser, 1993) were used.

According to the results of our experiments the yellow proved to be the most reliable colour for its monitoring. White and blue could be used with weaker, but still

reasonable effectiveness. Where *T. tabaci* and *Frankliniella occidentalis* are required to be monitored the application of the combined yellow and blue CSALOMON trap is advantageous.

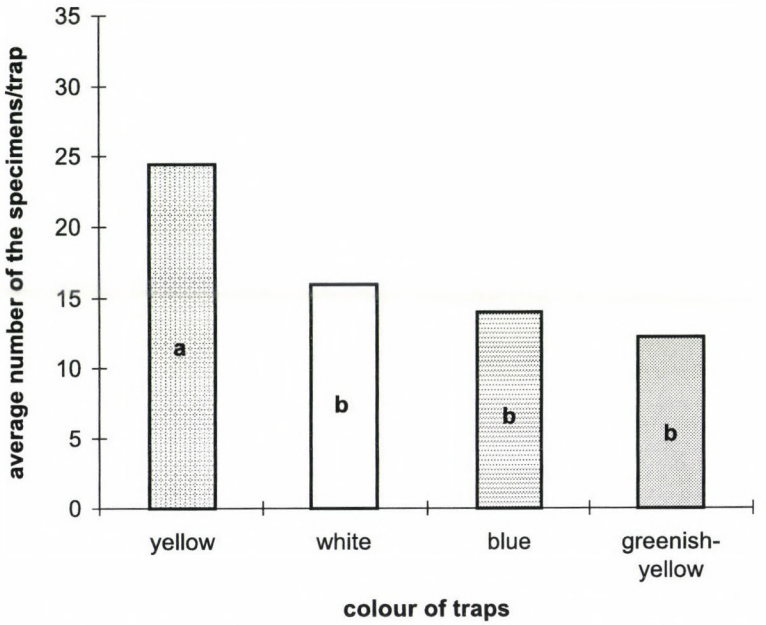


Fig. 3. Average number of *Thrips tabaci* specimens caught by the colour traps (Bököny, 1999) (Columns denoted by different letters are significantly different at a significant level $p < 0.05$)

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First Record of *Rhopalosiphum maidis* (Fitch) (Homoptera: Aphididae) in Slovakia

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During the autumn of 1999, an outbreak of *Rhopalosiphum maidis* (Fitch) was observed on barley volunteers at locality Nitra-Malanta (48° 19′ N, 18° 09′ E) in Slovakia. Barley plantation arose from dropped grain of spring barley harvested on July 20, 1999. The soil was not cultivated after the harvest. During the observation, in October, nearly 80% of tillers were in growth stage of heading and nearly 20% of tillers were in growth stage of booting. The abundance of *R. maidis* reached 61.13 and 67.37 individuals per main and lateral tiller respectively. *R. maidis* was not observed on maize fields in the vicinity of the infested barley plantation. The highest percentage of populations constituted larvae and nymphs. Alate females formed 6.7% of the *R. maidis* population. From 2116 aphids observed on 30 main tillers 97 (4.58%) and from 2283 aphids observed on 30 lateral tillers 153 (6.70%) were infected by entomophthoralean fungi *Entomophthora planchoniana* Cornu or *Erynia neoaphidis* Remaudière et Hennebert.

Keywords: *Rhopalosiphum maidis, Entomophthora planchoniana, Erynia neoaphidis.*

Rhopalosiphum maidis (Fitch) is a common aphid on maize in many countries of the world. It is usual on maize in the USA (Irwin and Thresh, 1988), south European countries like Portugal (De-Gouveia, 1984), France (Breniaux et al., 1976), or Spain (Pons et al., 1989; Fereres et al., 1990) and in Africa (Darwish and Ali, 1991). *R. maidis* was common on maize in Bulgaria (Grigorov, 1982) and Turkey (Bremer and Raatikainen, 1975).

Because *R. maidis* develops only partenogenetically, and it is not able to overwinter by eggs (Müller, 1961), it does not occur in colder regions of Europe. Its distribution in Europe is limited from 40° N to 45° N, accidentally up to 50° N (Knechtel and Manolache, 1944). In 1960, *R. maidis* was recorded for the first time in eastern Germany (Müller and Freitag, 1960). In England, *R. maidis* was first found in 1955 (Stroyan, 1955). *R. maidis* was also observed in Sweden (Ossiannilsson, 1959), Poland (Kania, 1962), Hungary (Milinkó et al., 1983; Mészáros et al., 1984) and Romania (Knechtel and Manolache, 1944; Manolache et al., 1968). Three main aphid species were found on maize at Rennes (northwest of France). The fourth species, *R. maidis,* was observed only once (Henry and Dedryver, 1989).

From 1997–1999, the population dynamics of aphids on maize plants was studied at Nitra-Malanta (48° 19′ N, 18° 09′ E) in Slovakia. The aim of the study was to explain how entomophthoralean fungi influence cereal aphid populations on maize. The results from the years 1997 and 1998 have already been published (Štalmachová and Cagáň, 1999). In October 1999, a new aphid species, *R. maidis,* was found on barley volunteers near the Nitra-Malanta locality.

Up to now, there has been no information about the occurrence of *R. maidis* in Slovakia. In this paper, circumstances surrounding the appearance of *R. maidis* in Slovakia are discussed.

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Materials and Methods

The field with barley volunteers, where *R. maidis* was found in 1999, was situated at Nitra-Malanta locality at altitude of 230 m, 1 km east of the experimental base of the Slovak Agricultural University in Nitra.

Barley volunteers rose from lost grains of spring barley harvested on July 20, 1999. The soil was not cultivated after the harvest. During the observation, in October, nearly 80% of barley tillers were in the growth stage of heading and nearly 20% of tillers were in growth stage of booting

At the barley volunteer field (variety Sladko, 0.5 hectare) where *R. maidis* was found, 30 plants were analysed for the occurrence of aphids. The main tiller and the first lateral tiller were taken for analysis from each plant. The alatae and apterous individuals of each aphid species were counted. The number of aphids infected by entomophthoralean fungi and parasitized aphids was recorded as well. The same procedure was conducted on 60 maize plants on maize field about 1 km distant from examined barley field.

Additional five maize fields were examined to find out if *R. maidis* occurs there, too.

To show if the population of *R. maidis* can well develop in Slovakia, the demographic structure of aphid population was investigated. Although, the demographic structure of other species found on barley volunteer field was made for comparison. Ten barley plants infested by aphids were put into the freezer for 5 hours. Then, aphids were collected from the plants by shaking, put in 70% alcohol and counted. Aphids of each species were sorted out into four groups; larvae, nymphs, apterous females and alate females.

The spectrum of entomopathogens infecting aphids on barley volunteers was identified by using the method of Papierok (1989). Each infected aphid was placed to a piece of damp cellulose inside a small Petri dish lid (diameter 35 mm). Then, the Petri dish lid was placed over a microscope slide. The damp conditions initiated the sporulation of fungus and the conidia were collected on microscope slide. After, the conidia were mounted in lactophenol-cotton blue. The fungal species was identified from the shape and size of conidia.

Results and Discussion

In 1999, the population dynamics of aphids on maize included two peaks. The first peak occurred during the second half of June and *Metopolophium dirhodum* (Walker) was the dominant species. The second peak was recorded at the beginning of September but aphid populations were much less numerous than in June. *M. dirhodum* was the dominant species again and 14% of the aphid population constituted *R. padi.*

The same aphid species were found at 5 other maize fields surveyed at different localities 15 kilometres around the locality Nitra-Malanta. *R. maidis* was found on maize plants neither during the growing season, nor in autumn.

This species was not usual on maize at colder localities. In Hampshire, England, on the maize crop, *M. dirhodum* and *R. padi* were found at densities that exceeded those

found on small-grain cereals and grasses at the same time of year. *Sitobion avenae* (F.), and *Macrosiphon euphorbiae* (Thomas) were found at relatively low densities, and *R. maidis* and *Macrosiphon festucae* (Theo.) were observed rarely (Hand and Carrillo, 1982). On the other hand, four aphid species, including *R. maidis, R. padi, S. avenae* and *M. dirhodum* were observed on barley plants during October 1999 in Slovakia. *Table 1* shows the numbers of individuals from each species. The average number of aphids per main and lateral tiller was 61.13 and 67.37 *(R. maidis),* 7.50 and 7.43 *(R. padi),* 1.67 and 1.30 *(S. avenae),* or 0.23 and 0.10 *(M. dirhodum),* respectively. The difference between numbers of aphids on the main and lateral tiller was insignificant (analysis of variance, Tukey test). The table shows that *R. maidis* had higher capacity to develop on barley plant than *R. padi. S. avenae* and *M. dirhodum* do not seem have any importance in autumnal damage of barley.

Table 1

Number of aphids (x±s) per main tiller and per lateral tiller of barley plant on October 13, 1999 (variety Sladko) and maize plants on September 30.
Locality Nitra-Malanta (48° 19′ N, 18° 09′ E). Altitude 230 m

	barley volunteers				maize	
	main tiller		lateral tiller			
	alatae	apterous	alatae	apterous	alatae	apterous
Rhopalosiphum maidis	1.3±1.92	59.83±59.80	0.6 ±0.95	66.7±48.58	0±0	0±0
Rhopalosiphum padi	0.1±0.40	7.40±10.47	0.03±0.18	7.40±6.72	0.03±0.18	0.13±0.78
Sitobion avenae	0±0	1.67±3.94	0.03±0.18	1.27±3.30	0±0	0±0
Metopolophium dirhodum	0±0	0.23±1.26	0±0	0.1±0	0±0	0.05±0.28

Data from bioassays of aphids collected from wheat and barley fields near Prosser, Washington State, showed that *R. maidis* reached high population densities only on late autumn barley (Halbert and Pike, 1985).

In studies on field populations of cereal aphids attacking small spring grains in South Dakota, *Schizaphis graminum* (Rond.), *S. avenae* and *R. padi* commonly attacked wheat, barley and oats, while *R. maidis* occurred only on barley (Kieckhefer, 1975). Infestation of barley with *R. maidis* occurred also in Magdeburg county, Germany (Dubnik and Thormeier, 1988). In the UK, 5 species of aphid commonly occurred on cereals between April and September. These species were *R. padi, M. dirhodum, S. avenae, Sitobion fragariae* (Wlk.) and *M. festucae. R. maidis* has been occasionally recorded on barley leaves (George and Plumb, 1982).

All information mentioned above supports the conclusion that in many countries, *R. maidis,* in spite of its name, develops much better on barley than on maize.

Several species of cereal aphids, including *R. maidis,* persist and reproduce parthenogenetically throughout the year in the southern areas of the USA. They persist less readily or not at all in the northern states and Canada where winters are cold and these areas are reinvaded annually by migrants from the south (Irwin and Thresh, 1988). Reports

from the USA show that the first migrants of *R. maidis* were observed during the first half of June (Post and Olson, 1961; Robinson and Hsu, 1963). The summer and autumn flight activity of cereal aphids was monitored in South Dakota, by operating a large suction trap continuously from 1 June to mid-November. *R. maidis* occurred in early August and in mid-September (Kieckhefer and Lytle, 1976). In southwestern Ontario the largest emigration of alatae from the maize plants was found in August and from about mid-September to mid-October (Foot, 1977). A similar situation occurs in Europe, only the migration of insects is relatively more limited by mountains. Mountain edges in America are oriented in the direction south-north and mountain edges in Europe are oriented in the direction east-west. Migrating insects need a suitable host plant for their next reproduction in new areas. In Slovakia, spring cereals are already harvested in July and maize is past flowering at the end of July. Except for maize or young cereals from seeds dropped during the harvest, other possible host plants should be grasses. *R. maidis* did not fare well on any of 12 species of grasses tested (Stoner and Kieckhefer, 1979). But, *R. maidis* was found on spring barley, *Echinochloa crus-galli* (L.) Beauv., *Festuca pratensis* Huds., and winter barley in eastern Germany (Geissler and Karl, 1987). *R. maidis* overwinters in a milder climate and is regularly reintroduced to central Hungary where it occurred on *Echinochloa cruss-galli* (Basky and Eastop, 1995). Matured maize plants in late summer and in autumn are probably not very suitable hosts for any aphid except for *R. padi* which is known as a species well developing on the oldest plant leaves (Pons et al., 1989). In Slovakia, winter cereals are usually sown during September–October. During the same months, young plants of winter cereals are emerging. In October it is probably too late to develop a new population of *R. maidis* on these plantations. But, we should mention that the weather in September and at the beginning of October was much warmer then usually. So, it seems that in a regular situation, without the summer development of barley plants, there is a little chance for the development of a strong *R. maidis* population in Slovakia.

The demographic structure of aphid populations on barley during autumn is shown in *Table 2*. Larvae and nymphs formed the highest percentage of populations. Alate females formed 6.7% of *R. maidis* population but only 0.6% of *R. padi* population. A

Table 2

Demographic structure of aphid population on barley plants on October 13, 1999 (variety Sladko). Aphids were collected from 10 randomly chosen plants. Locality Nitra-Malanta. Altitude 230 m

	n	Larvae % (n)	Nymphs % (n)	Alate females % (n)	Apterous females % (n)
Rhopalosiphum maidis	1472	52.6 (774)	38.2 (562)	6.7 (99)	2.5 (37)
Rhopalosiphum padi	342	59.9 (205)	34.5 (118)	0.6 (2)	5.0 (17)
Sitobion avenae	59	57.6 (34)	22.0 (13)	6.9 (4)	13.6 (8)
Metopolophium dirhodum	13	69.2 (9)	30.8 (4)	0.0 (0)	0.0 (0)

higher number of apterous females occurred in the population of *R. padi*. Females accounted for more than 20% of individuals of the *S. avenae* population. The results suggest that *R. padi* is more adapted to colder temperatures in autumn than *R. maidis*. The *R. padi* population still did not produce high number of alate females in October. This is not very surprising because *R. maidis* is the aphid of warm areas, whereas *R. padi* is usual pest of cereals especially in the countries of northern Europe (Andersson, 1977; Chiverton, 1986).

A higher ratio between females and immature forms in the population of *S. avenae* should indicate that production of young larvae is smaller than it is in the population of the other two species. The conditions for the development of *S. avenae* were probably less suitable than for both *Rhopalosiphum* species. Similarly, when *R. maidis* and *R. padi* were fed on resistant maize varieties, an increase was observed in the migration of apterous and in the incidence of alatae (Vereshchagina and Gandrabur, 1988).

From 2116 aphids observed on 30 main tillers, 97 (4.58%) and from 2283 aphids observed on 30 lateral tillers, 153 individuals (6.70%) were infected by entomophthoralean fungi. From the sample of 95 infected aphids, 70 were infected by *Entomophthora planchoniana* Cornu and 24 were infected by *Erynia neoaphidis* Remaudière et Hennebert (= *Entomophthora aphidis* Hoffmann). Mixed infection caused by both entomophthoralean species was found in one case. It seems that the same fungus species infect the aphid in different climatic conditions. *E. neoaphidis* caused the infection of *R. maidis* in Israel (Kenneth and Olmert, 1975) or Canada (Remaudière et al., 1978), *E. planchoniana* in Israel (Kenneth and Olmert, 1975).

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Book review

Vanneste, J. L. (ed.): Fire Blight. The Disease and its Causative Agent, *Erwinia amylovora.* CAB International, Wallingford, Oxon, 2000. pp. 370.

This book is about the fire blight, the most serious bacterial disease of apple and pears. It is the first multi-authored book on fire blight, written by 25 international experts, who critically reviewed the literature and shared their knowledge and experience on this disease.

The book represents the most up-to-date information available, written by some of the most influental scientists working on fire blight today. Most chapters contain information unpublished so far. After a short introduction about the disease written by the editor, it is divided into three sections. The first section contains important informations about the disease (epidemiology, distribution and economic importance of fire blight, genetic diversity and host range of *E. amylovora,* migration of bacteria in host plant tissues). The second section is about the causal agent, *E. amylovora:* its general characteristics, biochemistry, serology and its disease specific genes, *hrp* genes, harpins and exopolysaccharides. In the third part the authors discuss the difficult problems of fire blight control. They look at chemical control, problems associated with streptomycin resistance, potential and limitation of traditional breeding and transgenic plants, risk assessment systems and models, biological control and finally integrated management strategies for the control of fire blight in orchards and in nurseries. The book contains 26 coloured pictures about the symptoms of disease and a list of references is at the end of each chapter. This book is intended for scientists, graduate and post-graduate students working in the fields of bacteriology, epidemiology, disease control, plant breeding and molecular plant-microbe interactions. It is not only a reference book on the fire blight disease, its causal agent and methods of control, but it also provides a springboard for future studies on these subjects.

This book gives new hopes for an old dream: understanding how and why *E. amylovora* causes fire blight and how to control this disease.

G. Kazinczi

Akadémiai Kiadó, Budapest

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Differentiation of Isolates of *Neovossia indica* by RAPD-PCR and Clustering Based on Teliospore Morphology

B. SHARIFNABI*, D. K. AGARWAL and N. MITTER

Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi, India, 110 012

Teliospores morphology of twenty isolates of *Neovossia indica* collected from different geographical regions of India was studied by light and scanning electron microscopy. The data subjected to ANOVA showed significant difference at 1% level among the isolates. UPGMA analysis divided the isolates into two major clusters. Eleven isolates were also subjected to random amplified polymorphic DNA analysis (RAPD) after monosporidial production. UPGMA clustering of these divided the isolates into two major clusters. This clustering had no correlation with the clustering based on teliospore morphology. The cluster analysis is indicative of the fact that variability exists among various isolates belonging to different geographical parts of Northern India. Teliospore morphology is a variable character, therefore, it is imperative to use approaches involving DNA polymorphism for searching variability among fungal isolates.

Keywords: variability, karnal bunt, teliospore morphology, RAPD.

Karnal bunt or partial bunt of wheat, caused by *Neovossia indica* (Mitra) Mundkur [= *Tilletia indica* Mitra], was first reported from India on *Triticum aestivum* L. (Mitra, 1931). It has since been reported from Mexico, Afghanistan, Pakistan, Nepal, Iraq and Iran (Warham, 1986; Singh et al., 1989; Torabi and Jalalian, 1996). Infection of wheat grains has also been reported from Arizona and California, USA (Ykema et al., 1996).

Moderate temperature, high relative humidity or free moisture, cloudiness and rainfall during anthesis has been reported to favour disease development (Agarwal et al., 1976; Bedi et al., 1949; Mundkur, 1943).

Since Karnal bunt is seed borne disease in addition to being air and soil borne, strict quarantine regulations exist regarding movement of wheat into USA and other countries where Karnal bunt has not yet established. Due to several interceptions of teliospores in wheat shipments entering the USA from Mexico, it has become a significant concern to the USA wheat industry and USDA (Matsumoto et al., 1984).

Variability in pathogenicity, morphological, cultural, physiological, biochemical and isozyme analysis characteristics of *N. indica* have been studied (Bonde et al., 1985; Peterson et al., 1984; Singh and Singh, 1988; Sharma et al., 1998; Singh et al., 1998).

Historically, smut fungi have been described, classified and identified based on the host, characteristics of the sori and teliospores. It generally assumed that smut fungi are host-specific, but recent studies revealed that the determination of relationships between smut fungi is much more complicated. Many apparently distinct species are able

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to hybridize, but to what extent is unclear (Shi et al., 1996). Moreover, often there is very little variation in teliospore morphology useful for distinguishing species. In the recent studies additional morphological as well as molecular characteristics have been undertaken to elucidate the existence of variability among different isolates of *N. indica* prevalent in India.

Materials and Methods

Collection of isolates

Twenty samples of *N. indica* on wheat were collected from various parts of Northern India viz., Punjab, Haryana, Himachal Pradesh, Uttar Pradesh, Delhi and its vicinity during April, 1997 and April, 1999 *(Table 1)*. The teliospores of these samples were taken from infected heads and subjected to following investigations.

Table 1

Different isolates of *Neovossia indica* collected from various parts of Northern India

Isolate No.	Location	Date of collection	Isolate No.	Location	Date of collection
Ni1	Delhi	Apr, 97	Ni11	Yamuna Nagar, Haryana	May, 98
Ni2	HAU, Hissar, Haryana	Apr, 98	Ni12	Kashipur, U. P.	May, 98
Ni3	HAU, Hissar, Haryana	Apr, 98	Ni13	Muzaffarnagar, U. P.	May, 98
Ni4	HAU, Hissar, Haryana	Apr, 98	Ni14	Yamuna Nagar,Haryana	May, 98
Ni5	HAU, Hissar, Haryana	Apr, 98	Ni15	Roper, Punjab	May, 98
Ni6	PAU, Ludhiana, Punjab	May, 98	Ni16	Pant Nagar, U. P.	May, 98
Ni7	PAU, Ludhiana, Punjab	May, 98	Ni17	Batala, Punjab	May, 98
Ni8	Gurdaspur, Punjab	May, 98	Ni18	Bilaspur, U. P	May, 98
Ni9	Muzaffar nagar, U. P.	May, 98	Ni19	Muzaffar nagar, U. P.	May, 98
Ni10	Jalandhar, Punjab	May, 98	Ni20	HAU, Hissar, Haryana	Apr, 99

Morphological studies

I) Light microscopy: One hundred dried teliospores of each sample were first bleached in concentrated H_2O_2 for 30–40 min to enable their counting of the number of meshes per teliospore diameter and characteristics of reticulum. Samples were rehydrated in Shear's mounting fluid (Chupp, 1940) and observed at x2000 magnification with an Olympus BX50 microscope. Measurements for length, width of the teliospores and sterile cells, gelatinoid sheath, width and height of reticulum and length of apiculus were taken with the help of ocular micrometer .

II) Scanning Electron Microscopy: Air dried teliospores were dusted on small pieces of double-sided adhesive tape, mounted on specimen stub, sputter-coated with gold-palladium under vacuum, ca. 20 nm for 4.5 min, 7.5 mA. The specimens were then observed and photographed in a LEO 435 VP SEM, operated at 15 kv following the procedure of Vánky et al. (1997).

Production of monosporidial lines

Teliospores of *N. indica* are dikaryotic in nature and hence it is essential to obtain monosporidial haploid cells for molecular analysis. Teliospores from single sorus of each isolates were surface sterilized and dusted over Petri plates containing 15–20 ml sterile distilled water and incubated at 17 °C for 15 days. The floating teliospores were streaked on the surface of Potato Dextrose Yeast extract agar (PDA + 0.1% Yeast extract) with the help of a sterile pasture pipette and incubated at 17 °C. The whitish fungal colonies developed after 2–3 days of incubation and started producing sporidia after another 3–4 days. The monosporidial lines of these isolates were obtained by using chamber micromanipulator and kept at 17 °C in Potato Dextrose Yeast extract agar for about 3 weeks to produce pure colonies. After the formation of colony, the cultures were transferred to Potato Sucrose Broth (PSB) at 17 °C on an orbital shaker incubator at 120 rpm for 20 days and subsequently the mixtures of sporidia and mycelial growth in PSB from single sporidial cultures were harvested by filtration through Whatman filter paper No 41. These cultures were then rinsed with sterile distilled water and frozen at –20 °C. The frozen samples were used for DNA extraction.

DNA extraction

Because of the slow growth habit of *N. indica,* a modified procedure from the cetyltrimethylammonium bromide (CTAB) method of Shi et al. (1996) was followed. 0.2–0.3 g of compressed frozen mycelium of each isolate was grounded in pre-cooled sterile mortar and pestle using liquid nitrogen. The fine frozen powder was transferred to a sterile centrifuge tube. Pre-warmed at 65 °C DNA extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 1.4 M NaCl and 2% CTAB) was added to this and incubated in a water-bath at 65 °C for 1 h. After incubation an equal volume of chloroform/isoamyl alcohol (24:1) was added and mixed gently for centrifugation at 10000 rpm, 25 °C for 10 min. The aqueous phase was transferred to a new sterile tube and the DNA was precipitated with 0.6 volume of cold isopropanol and 0.1 volume of Sodium acetate (3M, pH 5.2) for 25 min at room temperature. DNA was pelleted by centrifugation at 10000 rpm for 10 min at 25 °C. The pellet was washed with cold 70% ethanol and resuspended in 100 ml dH_2O and frozen at –20 °C until needed for PCR amplification.

Purification of DNA

A stock solution of RNase-A was prepared @ 10 mg ml^{-1} in 10 mM Tris-HCl pH 8.0 and 15 mM sodium chloride. The solution was boiled for 10 min to destroy DNase. From the stock, 2µl of RNase-A was added to the crude DNA sample and incubated at 37 °C for 1 h. The DNA concentration of the samples and its purity was determined by taking ultraviolet absorbance at 260 nm and 280 nm in a spectrophotometer.

Random Amplification of Polymorphic DNA (RAPD) conditions

Twenty-five 10 mer oligonucleotides from sets P, S, M and N (Operon Technologies, Inc, USA) were used as single primers for the amplification of sequences. The PCR reaction was performed in a Gene-Cycler (Bio-RAD, USA), in a 25 µl volume

containing 4 µl of 1X reaction buffer (10 mM Tris-HCl, pH 8.3 and 50 mM KCl), 5 mM $MgCl_2$, 0.5 unit of Taq DNA polymerase, 100 µM each of dATP, dCTP, dGTP and dTTP (GENEI, Bangalore, India), 1 µM of 10 mer primer and 25 ng of template DNA. The amplification conditions were: initial step of denaturation at 94 °C for 2 min followed by 40 cycles of denaturation at 92 °C for 1 min, primer annealing at 37 °C for 1 min and extension at 72 °C for 2 min, followed by an extended elongation step at 72 °C for 5 min. Samples of 25 µl PCR products were mixed with 3 µl of 10X loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose, W/V) and spun briefly in a micofuge before loading (Sambrook et al., 1989). The amplification products were analysed on 1.2% agarose gel containing 0.2 µg ml^{-1} of ethidium bromide in 1X TAE buffer running at 60 volts for three hours and visualized under UV light and photographed on Polaroid 667 film under UV light. The DNA size marker used was λDNA double digested with *Eco*RI and *Hin*dIII.

Data analysis

1) MORPHOLOGICAL DATA ANALYSIS

The data obtained from morphological studies was analysed by two methods viz.,

I) Analysis of Variance:

The Analysis of Variance (ANOVA) was performed by using GLM procedure of PC SAS (SAS Institute, 1989). The mean comparison of the studied characters of the isolates was separately done using Duncan's Multiple Range Test (DMRT) .

II) Hierarchical Cluster Analysis:

The dissimilarity matrix was calculated from standardised morphological characters data using Euclidean measure of distance. Unweighted pair group method using arithmetic average (UPGMA) was selected to generate grouping (Sneath and Sokal, 1973).

2) RAPD DATA ANALYSIS

Each amplification product was considered as RAPD marker. Gels were scored on the basis of the presence (1) or absence (0) of each band for all isolates. All amplifications were repeated at least twice and only reproducible bands were considered for analyses. Jaccard's similarity coefficient values for each pairwise comparison between isolates were calculated and a similarity coefficient matrix was constructed. This matrix was subjected to unweighted pair-group method for arithmetic average analysis (UPGMA) to generate a dendrogram using average linkage procedure. All the numerical taxonomic analysis were conducted using software NTSYS – pc, version 1.80 (Rohlf, 1988).

Results

The teliospores of twenty isolates of *N. indica* were subjected to morphological characterisation for the parameters like, teliospore length and width, sterile cells length and width, gelatinoid sheath thickness and length of apiculus *(Table 2)*. One hundred teliospores were taken up from infected wheat heads to study each of the characteristic.

Table 2

Comparison of the mean of studied characters of *Neovossia indica* using Duncan's Multiple Range Test

Isolate no.	Spore length		Spore width		Sterile cell length		Sterile cell width		Meshes per spore diam.		Reticulum width		Reticulum height		Gelatinoid sheath		Apiculus	
	Mean	Rank	Mean	Rank	Mean	Rank	Mean	Rank	Mean	Rank	Mean	Rank	Mean	Rank	Mean	Rank	Mean	Rank
Ni1	30.00	D	27.78	BC	21.96	CD	17.75	GHI	8.93	F	3.83	AB	4.11	BC	5.11	CD	0.58	C
Ni2	28.47	E	26.16	E	19.45	E	16.19	K	8.56	KI	3.87	A	4.31	A	5.45	A	0.61	C
Ni3	30.89	CD	24.92	F	21.38	DE	17.52	GHI	8.47	FGH	3.80	AB	4.22	AB	5.19	BC	2.26	A
Ni4	32.07	AB	26.60	DE	24.42	A	18.96	CDE	8.61	GHI	3.79	ABC	4.3	A	5.36	AB	2.45	A
Ni5	28.28	EF	25.32	F	20.63	EFG	17.08	IJ	8.67	GH	3.74	BC	4.12	BC	5.06	CDE	0.95	BC
Ni6	26.79	G	24.09	G	21.24	DE	18.01	FGH	8.44	I	3.78	ABC	4.02	CDE	5.10	CDE	1.26	BC
Ni7	26.86	G	23.34	G	18.21	I	15.65	K	8.53	HI	3.67	CD	4.08	BCD	5.02	CDEF	1.42	B
Ni8	27.36	FG	23.89	G	19.80	GH	16.41	JK	8.78	FG	3.61	D	4.00	CDE	4.99	CDEFG	1.03	BC
Ni9	30.83	CD	26.31	DE	21.37	DE	17.14	IJ	9.20	F	3.37	E	3.79	F	4.77	HI	1.18	BC
Ni10	31.76	ABC	26.20	DG	23.25	B	19.69	BC	9.22	DE	3.31	EF	3.62	G	4.62	I	1.17	BC
Ni11	32.37	AB	29.39	A	24.34	A	20.81	A	9.41	CD	3.19	FG	4.11	BC	5.11	CD	1.33	BC
Ni12	31.94	ABC	27.07	CD	23.18	B	20.00	B	9.56	BC	3.21	FG	3.82	F	4.81	GH	1.01	BC
Ni13	31.82	ABC	28.41	B	22.82	BC	18.61	EF	9.72	AB	3.17	G	4.07	BCD	5.09	CDE	0.75	BC
Ni14	32.76	A	30.07	A	21.51	DE	18.74	EF	9.77	AB	3.15	G	4.11	BC	5.07	CDE	1.13	BC
Ni15	30.20	D	26.36	DE	20.89	EF	17.56	GHI	9.37	CDE	3.27	EFG	3.91	DEF	4.91	DEFGH	0.94	BC
Ni16	31.93	ABC	28.14	B	22.88	BC	19.56	BCD	9.62	AB	3.18	G	3.96	CDEF	4.93	DEFGH	1.09	BC
Ni17	31.57	BC	27.97	B	20.53	EFG	17.79	GHI	9.69	AB	3.14	G	3.63	G	4.60	I	0.87	BC
Ni18	30.38	D	26.17	E	20.53	EFG	18.34	EFG	9.66	AB	3.18	G	3.91	DEF	4.90	EFGH	1.04	BC
Ni19	31.88	ABC	28.12	B	19.97	FGH	17.32	HI	9.80	A	3.15	G	3.87	EF	4.86	FGH	1.04	BC
Ni20	32.25	AB	29.29	A	22.10	CD	18.87	DE	9.72	AB	3.16	G	4.11	BC	5.10	CDE	1.04	BC

The mean length of telispores varied from 26.76 μm for Ni6 to 32.76 μm for Ni14. The teliospores of Ni14 had the maximum width of 30.07 μm. The range of length and width for sterile cells was 18.4 μm to 24.34 μm and 15.65 μm to 20.81 μm, respectively. The teliospores did not show much variation as to the number of meshes per spore diameter as well as length and width of reticulation. The average thickness of gelatinoid sheath of all the isolates of *N. indica* was 4–7 μm. The data for apiculus length varied as the samples examined consisted of both young and mature teliospores. In young teliospores the length of the apiculus went up to 10 μm whereas in mature teliospores it was very small or even absent *(Fig. 1)*.

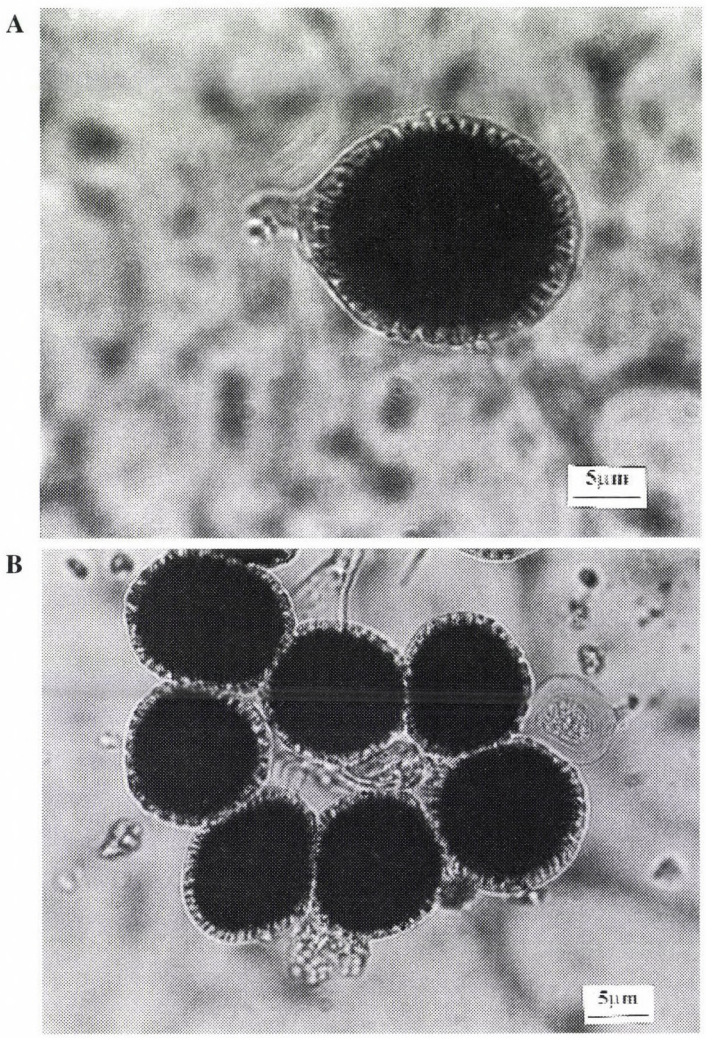


Fig. 1. Teliospores of *Neovossia indica* under light microscope
A) Mature teliospore with a short apiculus, X800; B) Mature, immature and smooth sterile cell, X800

Under SEM teliospores showed prominent thick projections of outer layer which were irregular blunt margins due to which the surface looked rough *(Fig. 2).*

The morphological characteristics were also evaluated in terms of correlation between various parameters *(Table 3).* It was observed that width and height of reticulum are negatively correlated with teliospore and sterille cells length and width. The thickness of gelatinoid sheath was found to be negatively correlated with teliospores length and width. The other parameters showed a positive correlation with each other.

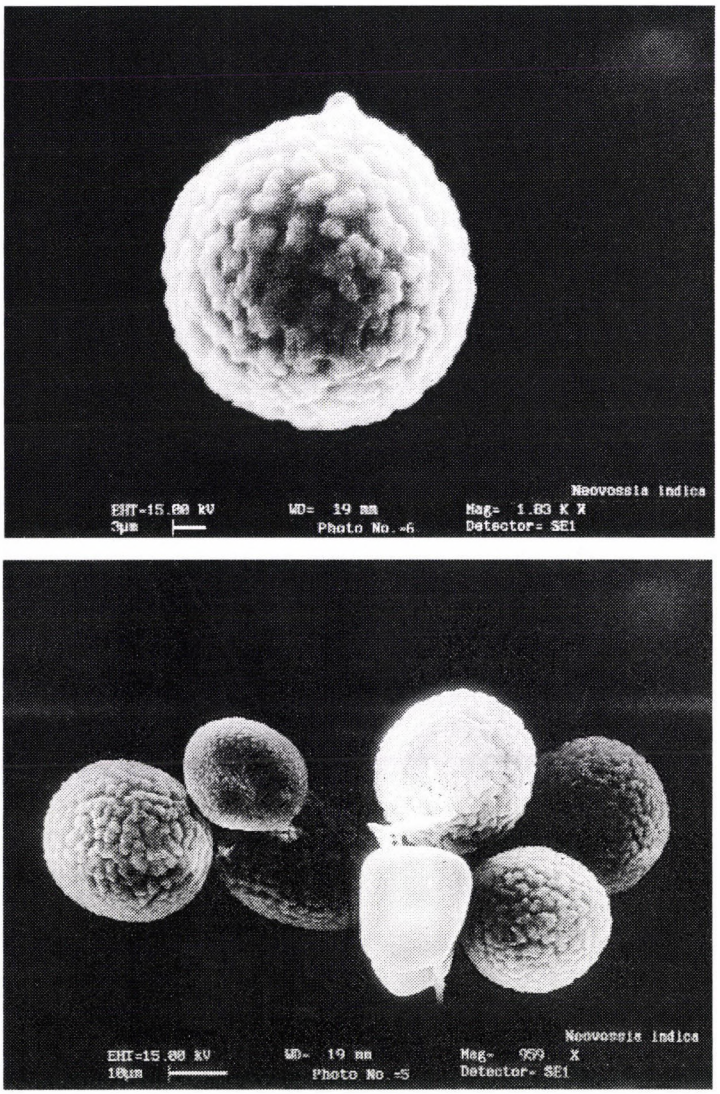


Fig. 2. SEM electron micrographs of surface ornamentation of teliospores
A) Mature teliospore with a short apiculus, X1830; B) Mature, immature and smooth sterile cell, X959

Table 3

Correlation of characters of different isolates of *Neovosia indica*

	Spore length	Spore width	S*. cell length	S*. cell width	Mesher per spore dim.	Reticulum width	Reticulum height	Gelatinoid sheath	Apiculus length
Spore length	1.00								
Spore width	0.842	1.00							
S*. cell len.	0.679	0.513	1.00						
S*. cell width	0.731	0.620	0.895	1.00					
Mesher / spore	–0.761	0.780	0.263	0.513	1.00				
Reticul. width	–0.675	–0.660	–0.250	–0.531	–0.951	1.00			
Reticul. hei.	–0.196	–0.049	0.023	–0.179	–0.488	0.588	1.00		
Gelatin shea.	–0.220	–0.057	0.031	–0.173	–0.509	0.607	0.980	1.00	
Apiculus	0.084	–0.331	0.313	0.106	–0.349	0.300	0.238	0.203	1.00

*Sterile cells

The ANOVA showed significant differences at 1% level for and between the isolates of *N. indica (Table 4).* The high coefficient of variance obtained for apiculus length is due to the fact that the observation varied from 0–10 μm owing to the presence of both young and mature teliospores.

Table 4

ANOVA of isolates of *Neovossia indica* for different characters of teliospores

Source of variance (S. V.)	Degree of freedom (df)	Mean Square (MS)**								
		Spore length	Spore width	S*. cell length	S*. cell width	Mesher per spore dim.	Reticulum width	Reticulum height	Gelatinoid sheath	Apiculus length
Isolates	19	374.64	354.15	263.54	177.02	24.12	8.30	3.77	4.54	21.25
Error	1980	12.53	8.07	11.26	7.04	0.47	0.17	0.30	0.38	5.03
Coefficient of variation %	–	11.60	10.61	15.59	14.67	7.49	12.02	13.84	12.45	192.63

*Sterile cells

**All sources of variance are significant at 1% level

The dendrogram obtained for morphological characters divided the *N. indica* isolates into two major clusters, "A" cluster consists of Ni14, Ni20, Ni13, Ni12, Ni16, Ni11, Ni15, Ni18, Ni9, Ni19, Ni17 and Ni10 and "B" cluster consists of Ni1, Ni3, Ni4, Ni7, Ni8, Ni5, Ni6 and Ni2. All the isolates from U. P. belonged to cluster "A" whereas isolates from Punjab and Haryana were distributed in both "A" and "B" cluster. The major clusters "A" and "B" were further divided into subgroups. These did not show any correlation as to the geographical distribution *(Fig. 3).*

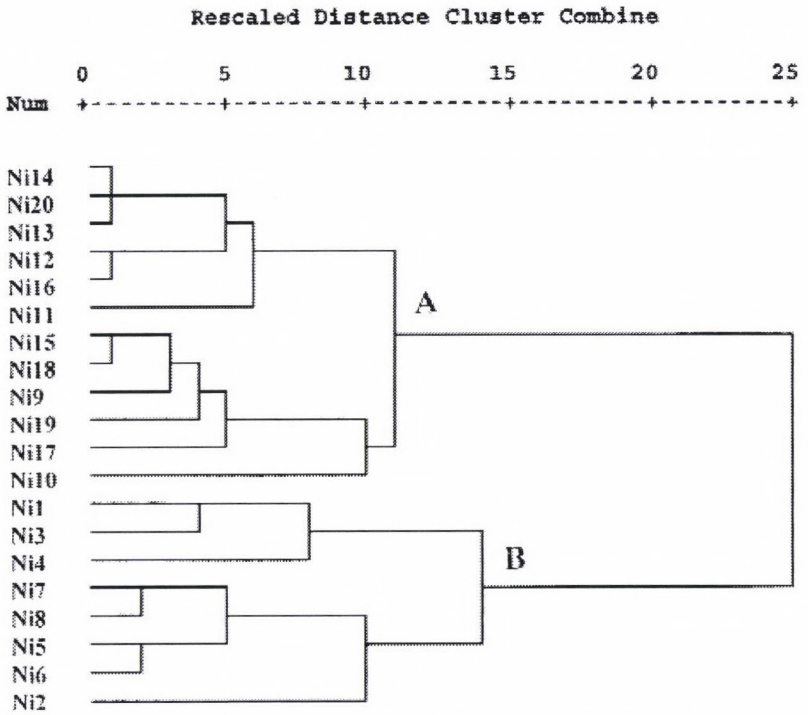


Fig. 3. Dendrogram of *Neovossia indica* isolates resulting from UPGMA based on different characteristics of teliospores

The isolates of *N. indica* were subjected to purification by obtaining monosporidial lines. The hyphae were then multiplied on potato sucrose broth to raise mycelial mass for DNA extraction. However, only 11 isolates of N. indica (Ni1, Ni4, Ni5, Ni8, Ni9, Ni10, Ni11, Ni12, Ni13, Ni18 and Ni20) yielded sufficient mycelium for DNA extraction and were further taken up for RAPD analysis.

Optimisation of RAPD markers

Good quality DNA of concentration 100 ng μl^{-1} was obtained from approximately 200–300 mg of frozen mycelial mass. Standardization of the PCR protocol was done by changing the variables like template DNA, Taq DNA polymerase and magnesium chloride. The optimum amplification was obtained by using the reaction mix having 25 ng template DNA, 0.5 unit Taq polymerase, 5 mM $MgCl_2$, 1 µM primer and 100 µM dNTP in reaction volume of 25 µl.

Twenty-five random 10 mer Operon primer from OPP, OPS, OPM and OPN series were screened. Seventeen primers were selected for doing RAPD analysis on the basis of reproducible and scorable polymorphic bands obtained *(Table 5).*

Table 5

Primers used and the number of total and polymorphic bands produced in *Neovossia indica* isolates

Primer	Sequence (3'–5')	Total bands	Polymorphic bands	Polymorphic percentage	Monomorphic bands
OPM06	CTGGGCAACT	9	9	100	0
OPM07	CCGTGACTCA	8	8	100	0
OPM13	GGTGGTCAAG	5	5	100	0
OPN02	ACCAGGGGCA	NA	NA	NA	NA
OPN05	ACTGAACGCC	NA	NA	NA	NA
OPN17	CATTGGGGAG	NA	NA	NA	NA
OPP01	GTAGCACTCC	NA	NA	NA	NA
OPP02	**TCGGCACGCA**	**18**	**17**	**94.40**	**1**
OPP03	**CTGATAGGCC**	**18**	**18**	**100**	**0**
OPP04	GTGTCTCAGG	12	12	100	0
OPP05	CCCCGGTAAC	12	12	100	0
OPP06	**GTGGGCTGAC**	**17**	**14**	**82.35**	**3**
OPP08	ACATCGCCCA	13	13	100	0
OPP09	**GTGGTCCGCA**	**18**	**18**	**100**	**0**
OPP10	TCCCGCCTAC	NA	NA	NA	NA
OPP16	CCAAGCTGCC	7	7	100	0
OPP19	GGGAAGGACA	11	11	100	0
OPP20	GACCCTAGTC	12	12	100	0
OPS01	CTACTGCGCT	4	4	100	0
OPS02	CCTCTGACTG	NA	NA	NA	NA
OPS03	CAGAGGTCCC	NA	NA	NA	NA
OPS05	TTTGGGGCCT	12	12	100	0
OPS06	GATACCTCGG	NA	NA	NA	NA
OPS08	TTCAGGGTGG	6	6	100	0
OPS09	TCCTGGTCCC	4	4	100	0
		174	**170**		**4**

RAPD analysis

A total of 174 bands were obtained for the 17 primers investigated out of which 170 were polymorphic. Primers OPP2, OPP3 and OPP9 amplified maximum number of polymorphic bands. The size of the fragments obtained varied from 0.5 to 4 kb *(Fig. 4).*

UPGMA cluster analysis

The data obtained from RAPD analysis of 11 isolates of *N. indica* with 17 primers was subjected to UPGMA analysis. A dendrogram for these 11 isolates was prepared using the similarity coefficient of RAPD marker *(Fig. 5).* The UPGMA clustering also divided the isolates of *N. indica* into two major groups. In cluster "I" there were two subgroups consisting of Ni1, Ni9, Ni8, Ni4 and Ni5 belonged to "Ia" and Ni10 and Ni1 belonged to "Ib". Cluster "II" also showed two major divisions "Ic" consisting only of Ni12 and "Id" having Ni13, Ni18 and Ni20. All the isolates in cluster "II"of RAPD analysis belong to cluster "A" of morphologically similar characters.

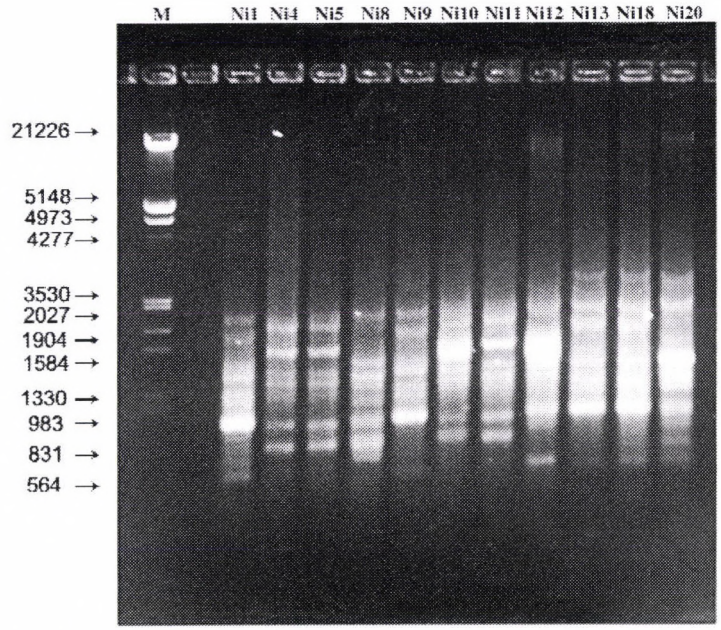


Fig. 4. RAPD analysis of 11 isolates of *Neovossia indica* with primer OPP-06. The products were separated in a 1.2% agarose gel stained with ethidium bromid. Lane M is λDNA double digested with *Eco*RI and *Hind*III

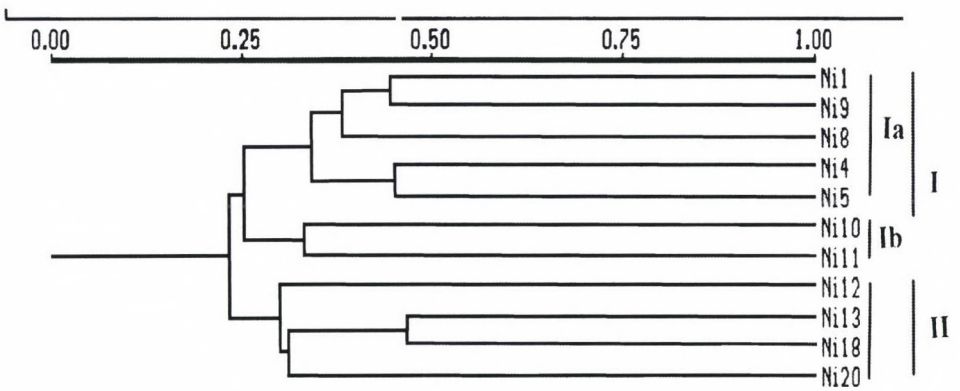


Fig. 5. Dendrogram from RAPD profiles of *Neovossia indica* isolates. Scale is distance by UPGMA method as implemented in NTSYS - pc

Discussion

The variability within species and generation of smut fungi is a product of genetic recombination during sexual reproduction. The progeny from a single generation may differ from each other and from the parent in virulence, host specificity and other characteristics (Fischer and Holton, 1957).

Physiological specialization in *N. indica* was first reported by Mitra (1931). He observed differences in the size of teliospores collected from Karnal, India and Peshawar, Pakistan, and speculated two races. Mitra (1935) regarded two different collections of *N. indica* as two physiological forms differing in teliospore size.

Munjal (1970) differentiated seven physiologic races on the basis of serology and their host reaction. The pathotypes could not be differentiated on the basis of the morphological studies as the shape and colour of teliospores in the samples resembled a lot.

Singh and Singh (1988) reported that since teliospore size is influenced by environmental variation, there are no large differences in size of the teliospores from different locations and varieties, hence it cannot be used as a differentiating character for the collections of *N. indica.* Observations of Bansal et al. (1984) indicated that average size of teliospores from various samples of wheat varieties collected from Punjab, Haryana and Uttar Pradesh differed significantly but the teliospore size from Himachal Pradesh and Jammu and Kashmir did not differ significantly within themselves in comparison to the type specimen. Apparently there is no difference in teliospore size between the type specimen and the samples studied. He concluded that the teliospore size is a slightly variable character and is influenced by the environmental factors.

In the present investigation the ultrastructure of the isolates was substantially similar but the clustering of the isolates based on various parameters divided them into two clusters "A" and "B". The teliospore size of isolates in cluster A was 26.2 – 30 x 30.2 – 32.7 µm and in cluster B of 23.3 – 27.7 x 26.6. – 32 µm. This clustering, however did not bear any correlation with the geographical distribution except for the fact that all the isolates from UP belonged to cluster A.

Peterson et al. (1984) did not find any significant differences among isolates of *N. indica* from India and Mexico for teliospore diameter.

SEM studies of teliospores of *N. indica* by Aggarwal et al. (1998) showed that episporium surface looked rough and the stretchability of perisporium did not keep pace with the advancing maturity.

Sharma et al. (1998) carried out pathogenicity test and isozyme analysis and grouped 5 distinct groups using pathogenicity and 2 groups based on the analysis of esterase and acid phosphatase isozymes. Singh et al. (1998) studied the biochemical variability of different isolates and found that the isolates varied considerably in their cellular fatty acid. Lipids analysis could not distinguish between the strains, but autoradiography allowed the strains to be distinguished clearly. No work has been done on the characterization of this important pathogen based on DNA polymorphisms.

DNA analysis of 11 isolates with 17 different random primers yielded 170 polymorphic bands which led to the division of isolates into 2 major clusters. The present in-

vestigation points to the fact that DNA polymorphisms can be exploited as an excellent and immensely valuable tool to study variability among isolates of *N. indica.* The cluster analysis indicates that variability exists among different isolates of *N. indica* between and within different geographical regions of Northern India. It points towards the fact that further investigation are needed to closely elucidate the variability spectrum of this important pathogen in relation to its pathogenicity.

The existence of variability among *N. indica* isolates is indicative of the fact that molecular marker technology along with pathotype analysis needs to be done in order to get a better insight into this important pathogen which at present is a potential quarantine threat to countries like Australia where it has not been reported so far. The data generated in the present investigation has for the first time utilized DNA polymorphisms to study variability among isolates of *N. indica* coupled with virulence analysis it can lead to the development of molecular markers which cab be very useful. The knowledge about the pathogen and the extent of variability spectrum forms the backbone to designing of management strategies. Also the patterns generated can be compared with isolates of *N. indica* from other parts of the world where it has been reported and Indian isolates to show the similarity percentage.

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Phenolic Compounds and Oxidative Enzymes in Cluster Bean Leaves in Relation to *Alternaria* Blight Severity

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The amount of polyphenoloxidase (PPO) increased with the increase in intensity of *Alternaria* blight up to 50 per cent in highly susceptible varieties (FS-277 and PNB), as compared to their respective healthy leaves (0 grade). At later stage, with the increase in disease intensity (>50%), enzymes activity started to decline. PO activity also increased with increasing intensity of *Alternaria* blight up to 50 per cent in FS-277, while in Pusa Navbahar, PO activity started to decline with the increase in disease intensity (>25%). Total phenolic compounds were observed to be higher in the varieties FS-277 and PNB with increase in *Alternaria* blight severity, as compared to control (0 grade). Ortho-dihydroxy phenols increased with increase in disease severity up to 25.00 per cent and with further increase in disease severity (>25%), these phenols started to decline. Flavanols were found to be reduced with increase in *Alternaria* blight severity, as compared to control (0 grade) in FS-277 and PNB tested in the present studies. Catalase was reduced significantly with the increase in *Alternaria* blight severity, as compared to healthy leaves (0 grade) in both highly susceptible varieties of clusterbean (FS-277 and PNB).

Keywords: cluster bean, *Alternaria* blight, phenolic compounds, oxidative enzymes.

Clusterbean [(*Cyamopsis tetragonoloba* (L.) Taub.)] commonly known as guar, has importance as an industrial crop of the third world, used as vegetables, feed and fodder in addition to a useful raw material for a wide range of industrial products (Joshi and Arora, 1993). Clusterbean foliage is subjected to a number of fungal diseases. Among these, *Alternaria* blight caused by *Alternaria cucumerina* var. *cyamopsidis* Rangaswami and Rao is a major foliar disease of clusterbean in Northern India. This disease is of great economic importance since it is responsible for reduction in the yield by 55.76–58.70 per cent under artificial epiphytotic conditions (Gupta, 1994). The information on biochemical parameters imparting resistance to *Alternaria* blight will be helpful for identification of sources of resistance of biochemical characters, which can be used as markers for screening of germplasm at an early stage for incorporating resistance against this disease.

Phenolic compounds are generally believed to be important in plant resistance mechanism and their accumulation is much higher in resistant genotypes than in the susceptible ones (Luthra et al., 1988; Sohal and Bajaj, 1993; Sindhan et al., 1996, Saharan et al., 1999). Oxidative enzymes such as polyphenol oxidase (PPO) and peroxidase (PO) contribute to resistance by restricting infection since they produce phenol oxidation products which are more toxic than phenols themselves. A positive correlation between the activities of these enzymes and disease resistance has also been observed in different field

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crops (Loebenstein and Linsey, 1966). Information on various biochemical parameters imparting resistance to *Alternaria cucumerina* var. *cyamopsidis* is lacking. Therefore, the present investigation was carried out to quantify the changes in total phenols, ortho-dihydric phenol, flavanols, activities of PPO, PO and catalase in healthy and diseased leaves (0–5 disease score) of highly susceptible varieties such as FS-277 and Pusa Navbahar (PNB).

Materials and Methods

Healthy (having no visible symptoms of *Alternaria* blight disease) and diseased cluster bean leaves (having 0–5 disease grade) of two highly susceptible (FS 277 and PNB) collected at 80 days after sowing, were freed of foreign material by washing thoroughly with running tap water followed by distilled water. Water drops from the leaf surface were removed by filter paper.

To 1.0 g fresh weight of finely chopped leaves, 4–5 ml of 0.01 M phosphate buffer, pH 7.0 was added and homogenised at 0–4 °C (Arora and Bajaj, 1981). It was strained through four layers of cheese cloth and then centrifuged for 20 min. at 10,000 g (0–4 °C). The resulting supernatant was used as enzyme extract. In each case, three separate extraction were made. PPO and PO were assayed according to the modified methods of Taneja and Sachar (1974) and Seevers et al. (1971), respectively. PPO and PO activity have been expressed as changes in 0.001 and 0.01 absorbance min^{-1} mg^{-1} protein, respectively. Catalase activity was measured by adopting the procedure of Sinha (1972) and expressed as μ moles of H_2O_2 utilized min^{-1} mg^{-1} protein. Suitable blanks in each case were run simultaneously. Soluble protein in the enzyme extract was precipitated by trichloroacetic acid (TCA) and determined according to Lowry et al. (1951).

Leaf samples left after extraction of enzymes, were dried at 60 °C in a hot air oven and ground in a micro-Willey mill. Total phenols and ortho-dihydric phenols were extracted and estimated according to the methods of Swain and Hillis (1959) and Johnson and Schaal (1952), respectively. Flavanols were estimated by the method of Balbaa et al. (1974).

Results and Discussion

Oxidative enzymes

The pattern of enzymatic activities of polyphenol oxidase (PPO), peroxidase (PO) and catalase in two highly susceptible varieties of cluster bean in relation to varying *Alternaria* blight severity (0–5 grade) is presented in *Table 1*. It has been found that PPO or PO activity increased with increase in intensity of *Alternaria* blight up to 50 per cent disease severity in variety, FS-277 thereafter the enzyme activity started to decline. There was significant increase in PPO activity at all disease severity levels as compared to 0 grade (control) in variety FS-277.

Table 1

Specific activity of oxidative enzymes in cluster bean leaves of highly susceptible varieties in relation to *Alternaria* blight severity

Disease severity (%)	FS-227			PNB		
	Polyphenol oxidase*	Peroxidase** (Units×10^2)	Catalase*** (Units×10^2)	Polyphenol oxidase*	Peroxidase** (Units×10^2)	Catalase*** (Units×10^2)
0	1.49	27.57	20.25	1.80	19.19	11.93
1.0–5.0	1.84	34.17	14.60	1.92	47.39	7.10
	(23.49)	(23.94)	(–27.90)	(6.67)	(146.95)	(–40.48)
5.1–10.0	1.92	103.95	13.90	2.04	131.76	5.85
	(28.85)	(277.04)	(-31.36)	(13.33)	(586.61)	(–50.96)
10.1–25.0	2.02	116.46	10.04	2.07	164.99	3.63
	(35.57)	(322.41)	(–50.42)	(15.00)	(759.77)	(–69.57)
25.1–50.0	2.05	397.71	10.01	2.63	94.69	1.44
	(37.58)	(1342.55)	(–50.57)	(46.11)	(393.43)	(–87.93)
>50.0	1.97	47.73	9.96	1.43	56.04	0.87
	(32.21)	(73.12)	(–50.81)	(–20.55)	(192.27)	(–92.71)
CD at 5%	0.12	22.22	2.17	0.14	0.36	1.02

Unit* – Change in 0.001 OD min-1 mg-1 protein; **change in 0.01 OD min^{-1} mg^{-1} protein;
*** µmol of H_2O_2 utilized min^{-1} mg^{-1} protein
Values in parentheses indicate per cent deviation from control (0 grade)

It is obvious from *Table 1* that maximum significant increase (37.58%) in PPO activity was observed in samples having 25–50 per cent *Alternaria* blight severity, as compared to 0 grade (free from disease). PO activity increased significantly in samples having more than 5 per cent disease severity, as compared to 0 grade (control) in variety FS-277. However, maximum significant increase (1342.55%) was observed in diseased leaves of variety FS-277 having 25.0–50.0 per cent disease intensity as compared to control (0 grade). The activity of catalase was reduced with the increase in disease severity, as compared to control (0 grade). There was significant reduction in catalase activity with all disease intensity levels over control. However, maximum significant decrease (50.81%) was found in leaves having >50.0 per cent disease severity, as compared to control (0 grade). Similar results were obtained with PPO, PO and catalase activities in variety PNB except for PO which started to decline after increase in disease severity beyond 25.0 per cent. Maximum significant increase of 46.11 per cent in PPO activity was observed in PNB leaves having 25.0–50.0 per cent disease intensity as compared to control (0 grade). However, maximum significant increase in PO activity of 759.77 per cent was found in PNB leaves having 10.1–25.0 per cent disease intensity over control. However, activity of catalase was reduced with increase in disease intensity even beyond 50.00 per cent, as compared to control. It was reduced by 92.71 per cent in *Alternaria* blight infected leaves (>50.00% intensity) over control *(Table 1).*

These results are in agreement with earlier observations on cluster bean in relation to *Alternaria* blight severity (Anonymous, 1997). The decline in PPO and PO activity after a certain level may be due to the death of infected plant tissues. Oxidative enzymes (PPO and PO) were considered to confer resistance by oxidation of phenolic compounds to quinones which latter are toxic to the invading fungi (Farkas and Király, 1962; Arora and Bajaj, 1985; Lukose et al., 1990). PO activity was many times higher as compared to PPO activity and increased markedly in response to infection in both varieties (FS-277 and PNB) in the present studies. Similar results were obtained in cluster bean leaves infected with bacterial blight disease (Luthra et al., 1988). These results suggested that peroxidase contributes more to resistance as its activity was several folds higher than the PPO activity. Peroxidase activity has been reported to be associated with deposition of lignin and suberin in plant cells (Van Fleet, 1962) and it also acts as an oxidising agent of aromatic compounds like coumarin, phenylaline and polyphenols (Loebenstein and Linsey, 1966).

Phenolic compounds

It is clear from *Table 2* that there was increase in total phenols in both varieties (FS-277 and PNB) with increase in *Alternaria* blight intensity, as compared to control (0 grade). Maximum significant increase of 36.41 and 59.53 per cent was found in *Alternaria* blight infected leaves with more than 50.00 per cent disease intensity as compared to control in varieties FS-277 and PNB, respectively. Ortho-dihydric phenols increased with increase in disease intensity up to 25.00 per cent over control after that it increased over control but decreased beyond 25.00 per cent disease severity as compared to diseased leaves of both the varieties having 10.00–25.00 per cent disease intensity. Maximum significant increase of 72.39 and 94.28 per cent in ortho-dihydric phenols was observed in *Alternaria* blight infected leaves (10.0–25.0% intensity) over control (0 grade) in varieties namely, FS-277 and PNB, respectively.

It can also be revealed from *Table 2* that flavanols reduced in *Alternaria* blight infected leaves in both varieties, as compared to healthy leaves (0 grade) with increase in disease intensity. Maximum significant decrease of 44.36 and 44.54 per cent was observed in leaves having more than 50.00 per cent disease intensity, as compared to control in varieties, FS-277 and PNB, respectively.

This may be attributed to the increased local synthesis of phenolics at the site of infection in order to check further spread of the disease. The increase in phenolics in response to infection suggested a positive role of phenolics in resistance of clusterbean plants to *Alternaria* blight disease. The post-infectional increase in total phenols is in agreement with the findings of Luthra et al. (1989) and Anonymous (1997) in clusterbean infected with bacterial blight. The post-infectional increase in ortho-dihydric phenols is also in agreement with the findings of Sindhan et al. (1996) in clusterbean infected with bacterial blight. However, the decrease in ortho-dihydric phenols after disease severity >25.00 per cent may be due to degeneration or death of tissues.

Similar results were obtained by Sangwan et al. (1996) in cowpea leaves infected with the yellow mosaic virus. Phenolic compounds are often considered to play a major role in disease resistance. They are easily oxidized to highly reactive quinones which are

Table 2

Phenolic compounds (mg g^{-1}, dry weight basis) in cluster bean leaves of highly susceptible varieties in relation to *Alternaria* blight severity

Disease severity (%)	FS-227			PNB		
	Total phenols	o-dihydric phenols	Flavanols	Total phenols	o-dihydric phenols	Flavanols
0	39.00	1.92	7.71	27.33	2.45	11.54
1.0–5.0	41.80	1.97	6.61	32.00	2.86	9.24
	(7.18)	(2.60)	(–14.27)	(19.50)	(16.73)	(–19.93)
5.1–10.0	46.20	2.63	5.64	33.63	3.50	10.51
	(18.46)	(36.98)	(–26.85)	(23.05)	(42.86)	(–8.92)
10.1–25.0	48.60	3.31	4.55	34.40	4.76	17.62
	(24.61)	(72.39)	(–40.98)	(25.87)	(94.28)	(52.68)
25.1–50.0	50.67	2.09	4.37	37.50	3.62	10.51
	(29.92)	(8.85)	(–43.32)	(37.21)	(47.75)	(–8.92)
>50.0	53.20	1.78	4.29	43.60	2.28	6.40
	(36.41)	(–7.29)	(–44.36)	(59.53)	(–6.94)	(–44.54)
CD at 5%	4.83	0.48	2.19	2.74	0.59	5.20

Values in parentheses indicate per cent deviation from control (0 grade)

effective inhibitors of sulfhydryl groups of enzymes, thereby preventing metabolic activities of host or parasitic cells, as has been first proposed by Farkas and Király (1962) and then by Thind et al. (1981). Varying levels of bacterial blight infection in clusterbean leaves resulted in a decrease in catalase activity as reported by Chaudhary and Lodhi (1981). Sangwan et al. (1996) also obtained similar results in cowpea leaves infected with yellow mosaic virus. In the present study, healthy leaves (0 disease grade) had higher catalase activity which indicated its role towards resistance. However, the exact role of catalase in resistance or susceptibility of plants towards pathogen is still controversial.

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A Simplified Method of DNA Extraction for Identification of *Fusarium graminearum* Group 2 Isolates by PCR

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Fusarium graminearum (teleomorph: *Gibberella zeae*) is widely spread pathogen of cereals and maize. Fast and reliable determination of this parasite and analysis of its DNA molecules is possible by amplification in polymerase chain reaction (PCR). DNA isolation is an operation necessary for amplification by PCR that takes a lot of time and increases possibility of sample contamination. Those are the reasons that DNA isolation needs to be simplified. A simplified procedure of DNA isolation from *G. zeae* perithecia was presented in this work.

Keywords: disease diagnosis, *Fusarium graminearum, Gibberella zeae,* plant disease, polymerase chain reaction.

Species of the genus *Fusarium* are widely spread fungi that cause crown, foot and root rot, head scab of wheat as well as root, stalk and ear rot of maize (Sutton, 1982; Gilbertson et al., 1985; Krüger, 1989; Parry et al., 1995; Vigier et al., 1997). An infection in a stage of seed germination causes death. In a later stage of plant development it causes a decrease of vascular system functioning and a premature maturation of seed. Ear rot of maize and wheat head scab in addition to yield decrease have very negative influence on product quality because of mycotoxins production in infected grain (Chelkowski and Manka, 1983; Snijders, 1990; Szécsi 1990; Visconti et al., 1990; Perkowski et al., 1997).

Two distinct populations, Group 1 and Group 2, have been recognized within *F. graminearum* (Francis and Burgess, 1977). Strains of Group 1 cannot form perithecia neither in nature nor *in vitro* (they are heterothallic) and they cause dryland foot rot of wheat. Recently, Aoki and O'Donnell (1999 a, b) described *F. graminearum* Group 1 as a new species *F. pseudograminearum* O'Donnell and Aoki, sp. nov. and succeeded to obtain heterothallic production of perithecia from the maiting experiments among seven strains of *F. pseudograminearum*. Based on the morphology of perithecia, asci and ascospores they described it as a new species *Gibberella coronicola* T. Aoki and O'Donnell, sp. nov. Isolates of Group 2 always form perithecia in nature and *in vitro* (they are homothallic) and cause diseases of aerial plant parts such as head blight of wheat, stalk and ear rot of maize.

PCR is a useful method for assessing of genetic diversity within a fungal species – isolates obtained from various hosts, tissues of organs, locations as well as for analysing

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of phylogenetic diversity of *Fusarium* species (Nicholson et al., 2000; Yli-Mattila et al., 2000). More recently, PCR-based marker systems have been developed to allow detection of the species most frequently associated with the SBD (stem base disease of wheat and barley) complex within plant tissue (Turner et al., 1999) and it does not need previous fungus isolation from host tissue (Schlacht et al., 2000).

Extraction of DNA from a sample is one of the most time consuming steps in a PCR analysis. The inhibitors in the samples frequently cause PCR inhibition. That is a reason for application of more complicated, time consuming methods for DNA extraction, that provide samples with low amounts of inhibitors. These methods usually assume: grinding of mycelium; sample purification with phenol, chloroform and isoamyl alcohol; precipitation with ethanol, etc. (Möller et al., 1992; Shi et al., 1996; Doohan et al., 1998). Simple boiling of mycelium can be applied as an alternative way for DNA extraction (Schilling et al., 1996). However, mycelium contains compounds that can cause inhibition and missing of PCR.

A simplified method of DNA extraction from fungi *F. graminearum* Group 2 was examined in this work. It assumes DNA extraction from perithecias of perfect stage of *F. graminearum* Group 2 *(Gibberella zeae).* DNA extracted on this way can be used for amplification in PCR and analysis of obtained PCR product for various purposes (pathogen identification, DNA polymorphism). Possibility for amplification of so extracted DNA in a PCR reaction was examined in this work. It was performed in a reaction, which was already developed for species identification (Schilling et al., 1996). *F. graminearum* sequence specific amplification was applied to test success of amplification. It was shown that possible inhibitors did not make significant influence on reaction.

It has to be mentioned that a group of scientists already developed a method for detection and as well as quantification of *F. graminearum* (Nicholson et al., 1998). For species detection they used sequences which were isolated and sequenced by themselves from a RAPD product (random amplified polymorphic DNA assay) (Williams et al., 1990).

Materials and Methods

Perithecia of *G. zeae* were taken from stalk of maize and wheat or from nutrient medium – carnation leaf agar (CLA) *(Table 1).* Isolates of *F. graminearum* Group 2 were obtained by standard isolation procedure on PCNB at 25 °C (Bagi, 1999). Pure cultures of isolates were grown on CLA under a 12 h light/12 h dark regime of near-UV (black light: Phillips TLD 36 W/08) and 20 °C (Burgess et al., 1994) to obtain fungi perithecia. Isolates of *F. graminearum* (Group 2) were identified according to morphological characteristics of conidia and homothallic production of a *G. zeae* teleomorph on medium and plant host.

Isolation of DNA

Isolation of DNA molecules was performed from perithecia of *G. zeae (Table 1)* that were taken from carnation leaf agar (CLA) and/or maize and wheat stalk. Eleven perithecia were taken from each sample, placed on microscope slides and ground with

other slides. Each sample was washed off the slides with 100 µl double-distilled H_2O into microtubes and incubated at 100 °C for 15 minutes.

Purified DNA molecules of *F. graminearum* (Group 2), provided by Dr. Evelyn Möller, were used as a positive control.

Table 1

Samples that were processed in the experiment

Code of sample	Host	Organ	Location	Origin of perithecia	Ordinal numeral in electrophoresis
Paz 2,1	Wheat	Stalk	Pazova	CLA	1
Neš 6	Corn	Ear	Nestin	CLA	2
B.M. 4	Corn	Ear	Backi Monostor	CLA	3
St341	Corn	Stalk	Krnjesevci	Host	4
St539	Corn	Stalk	Velika Plana	Host	5
StConzt	Corn	Stalk	Zemun Polje	Host	6
St704	Corn	Stalk	Zemun Polje	Host	7
1D	Wheat	Root	Rimski Sancevi	CLA	8
1	Wheat	Stalk	Zemun Polje	Host	10
2	Wheat	Stalk	Zemun Polje	Host	11
3	Wheat	Stalk	Zemun Polje	Host	12
Control	–	–	–	–	9, 13

PCR

Polymerase chain reaction was performed in 50 µl volume. Reaction mixture contained 2 units of *Taq* polimerase, 200 µM of each dNTPs, 25 pmol each primer, 25 µl of DNA solution. Reaction was performed in 1x buffer for PCR (67 mM tris-HCl, pH 8.4; 2.5 mM $MgCl_2$; 16.6 mM of ammonium sulfate; 0.01% triton X-100).

Sequence of oligonucleotide primers were (Schilling et al., 1996):

UBC85F 5' – GCAGGGTTTGAATCCGAGAC

UBC85R 5' – AGAATGGAGCTACCAACGGC

Temperature and timing profiles of PCR

PCR was processed in 3 waterbath at different temperatures. Test tubes were manually transferred from one to other waterbath after a determined time. Temperatures were: initial denaturation – 2 minutes at 95 °C, denaturation – 60 seconds at 95 °C, annealing – 60 seconds at 61 °C, extension – 60 seconds at 72 °C and final extension – 5 minutes at 72 °C.

F. graminearum sequence specific amplification was applied to test success of amplification (Schilling et al., 1996).

Analysis of PCR product

Analysis of PCR product was processed with electrophoresis in 1.5% agarose gel (Sambrook et al., 1989). DNA of plasmid pTZ18R digested with restriction enzyme Bsp143I was used as a marker.

Results and Discussion

Expected amplification product should be 332 bp long. This product was obtained from every sample but St341 *(Fig. 1, lane 4).* According to obtained results, PCR assays confirmed that about 91% of perithecia belonged to *G. zeae.* Isolation of DNA from perithecia gave more reliable results than isolation of DNA by simple boiling of fungi mycelium. Our preliminary studies, when we isolated DNA by boiling of mycelium, showed that product of reaction was missed in more than 50% of samples. Sometimes, sample dilution can decrease concentration of inhibitor substances and it makes possible to obtain an expected product. However, in this case, decreasing of reaction inhibitor concentration by DNA dilution did not give any improvement.

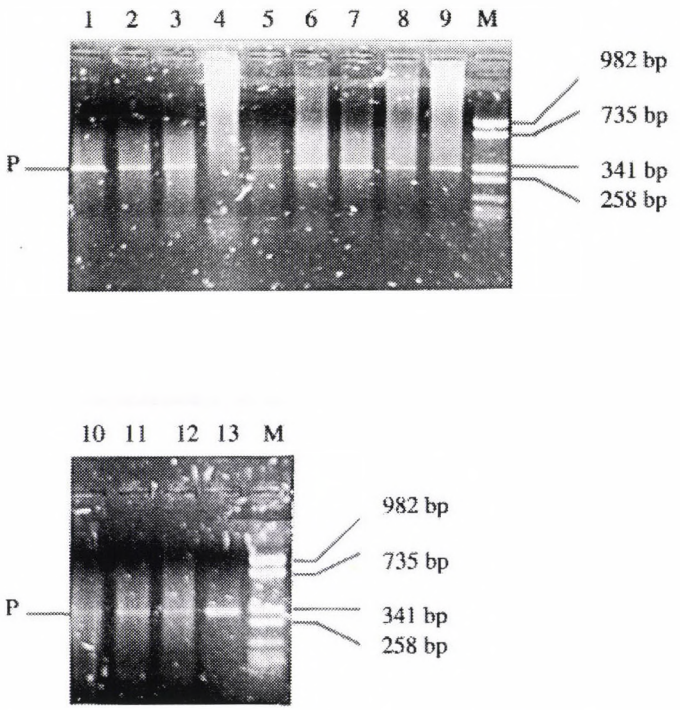


Fig. 1. Electrophoresis gel of PCR product taken on a transilluminator under UV light (lanes 1–13 are samples; M – marker; P – PCR product 332 bp long). All lines contains PCR product 332 bp long, except line 4

Samples 1–8 *(Table 1; Fig. 1)* collected in fall 1998 were analyzed in May 1999. These samples with developed perithecia (from which DNA was isolated) were analyzed repeatedly and just 1 sample in average did not give expected product. However, in September 1999 the same samples (1–8) did not give any product in a repeated analysis.

Conditions of sample storage were changed during the period from June to September 1999 and we assume that it was the reason for missing of the PCR product. Until June 1999 all samples were stored at +4 °C and after that period at room temperature. Microscopic investigations of the samples showed that asci with ascospores were released from perithecia because of the temperature changes. Similar appearance was recorded with isolates Paz 2,1; Nes 6 and B.M.4, grown on CLA media in August 1999 that were analyzed in September of the same year.

DNA from perithecia of samples 10–12, 3 months old, originating from wheat foot, gave expected PCR product *(Fig. 1)*. This experience shows that it is very important to check if perithecia contain differentiated asci with ascospores for successful analysis.

Complicated procedure for DNA isolation from mycelia that assume sample purification with phenol, chloroform, ethanol precipitation, etc., allows more reliability (Möller et al., 1992). However, this mentioned method of DNA isolation is time consuming, increases consumption of chemicals and expense of analysis. The main shortcoming of this procedure is an increased possibility of sample contamination, especially if a large number of samples is processed. Contamination is possible among the samples as well as between samples and an external source of contamination. Increased risk of contamination is a result of larger number of operations in purification of DNA molecules than in other procedures.

DNA can be isolated by simple boiling of mycelium, but concentration of PCR inhibitor after this procedure can be high and cause missing of reaction. Molecules of DNA isolated from perithecia by simplified method described here, contain less PCR inhibitor and method is reliable *(Fig. 1)*. However, amplification of *F. graminearum* Group 2 DNA is possible only if perfect stage of fungus is developed.

To decide which described method of DNA isolation is the most suitable depends of type of samples and occasions under which samples have to be processed. It is very important to take care of storing conditions of samples and differentiation of asci with ascospores in perithecia. A stereomicroscope observations are useful for detection of asci and ascospores differentiation in perithecia and their selection for DNA isolation.

Described simplified method of DNA extraction will enable correct and fast identification of *F. graminearum* Group 2 teleomorph that form on diseased root, stalk, ear/head of maize and wheat, to follow their development during the growing season and to study DNA polymorphism of samples with different origin.

In consideration of fact that several phytopathogenic fungi cause wheat and maize diseases (Hill et al., 1983; Windels and Holen, 1989; Rossi et al., 1995) we think that the simplified method of DNA extraction from perithecia of *G. zeae* could be useful for PCR identification of other phytopathogenic fungi that form perithecia on these hosts [(e.g. *Gaeumannomyces graminis* (Sacc.) Arx and Olivier, *Calonectria nivalis* Schaffnit and others on wheat stalk)].

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Variation in Sensitivity to Fungicides among Isolates of *Alternaria solani* Causing Tomato Leaf Blight Disease

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Isolates of *Alternaria solani* causing tomato leaf blight disease were collected from twenty conventional tomato growing regions and their pathogenicity established. A wide variation existed among the isolates in terms of virulence on tomato plants. Sensitivity of the isolates to different fungicides was evaluated based on mycelial growth on fungicide-amended culture media and spore germination after fungicide treatment. All the chemicals tested were effective in reducing the mycelial growth and spore germination of *A. solani* isolates however, mancozeb (0.2%) followed by captofol (0.2%) were more effective. Virulent isolate recorded more mycelial growth and spore germination. A positive correlation was found between the virulence of isolates and ability to grow and sporulate after exposure to fungicides.

Keywords: Tomato, *Alternaria solani*, fungicides, sensitivity.

Use of chemical fungicides remains to be the important tool in management of crop diseases. It becomes the first priority in the absence of a resistant variety or when resistance in the variety fails (Gangawane, 1997). Fungicidal sprays are widely used over a long time for the management of leaf blight caused by *Alternaria solani* (Ell. and Mart.) Jones and Grout, a serious disease of tomato. The effective fungicides include mancozeb (Abol-Wafa and Kamara, 1975; Romann et al., 1982; Sinha and Prasad, 1991), copper oxychloride (Kamaleshmathur and Shekawat, 1986), Bordeaux mixture (Shayam and Sharma, 1974), captofol (Chaudhari and Patel, 1987), Zineb (Choulwar and Datur, 1988). The convention method of using chemicals is although effective in reducing the intensity of the disease, in future use of chemicals is going to be gradually dispensed with (Babu et al., 2000a). The prime factor being the development of fungicidal resistance in pathogens (Hewitt, 1998). It is important to monitor fungicide resistance so that any shifts in fungicide sensitivity in fungal populations can be detected to implement alternate disease control strategies. Mycelial growth and spore germination tests are the biological methods commonly employed to test fungicide resistance. The present study was undertaken to determine the variability among *A. solani* isolates of tomato, as virulence and sensitivity to fungicides.

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Materials and Methods

Collection of A. solani *isolates*

Tomato leaves showing typical symptoms of leaf blight disease were collected afresh from twenty conventional tomato growing areas of Tamil Nadu State, India. The diseased leaf tissue was cut into small bits using sterilized scalpel, surface sterilized in 0.1 per cent mercuric chloride for 30 sec and washed in repeated changes of sterile distilled water. The leaf bits were placed on to sterilized potato dextrose agar (PDA) medium in Petri dishes under aseptic conditions and the plates were incubated at room temperature (28±2 °C). The fungal growth was purified by single spore isolation technique and the cultures were maintained on PDA.

Virulence test on tomato plants

Tomato plants of susceptible cultivar PKM1 was raised in pots (30 cm) each filled with 3 kg of uniform soil mixture. When the plants are 75 days old, they were sprayed with conidial suspension ($5X10^5$ conidia/ml) prepared in sterile water from 7-day-old culture of different isolates of *A. solani*. Water congestion was provided 24 h before and after inoculation by covering the plants with polythene sheet sprayed with water. The plants were maintained in glass house. Plants sprayed with distilled water served as control. Due care was taken to avoid drift effects of spaying of inoculum of the individual isolates on the respective set of plants. Three replications were maintained for each isolate. Disease intensity was recorded 10 days after inoculation. Ten leaves at random were selected in each replication and grades were awarded based on the per cent leaf area diseased as per the standard grade chart of 0–9 scale (Babu et al., 2000a). Per cent disease index (PDI) was worked out using McKinney's (1923) formula

$$PDI = \frac{\text{Total sum of numerical ratings}}{\text{Total number of leaves observed}} \times \frac{100}{\text{Maximum grade}}$$

Evaluation of fungicides against A. solani *isolates*

Six fungicides viz., mancozeb (0.2%), copper oxychloride (0.25%), chlorothalonil (0.2%), captofol (0.2%), aureofungin (antifungal antibiotic) 200 ppm and validamycin (0.1%) were tested against *A. solani* isolates *in vitro* by mycelial growth and spore germination assay.

The required amount of each chemical was weighed and stock solution of each fungicide was prepared in sterile distilled water. The desired amount of fungicide suspension was added to autoclaved potato dextrose agar and mixed thoroughly before plating. Twenty ml of this fungicide amended medium was then poured into each plate and inoculated with 9 mm mycelial disc of each isolate separately and incubated. Medium without fungicide served as check. The colony diameter was measured 10 days after inoculation.

One drop of the fungicidal suspension was placed into the cavity slide and allowed to air dry. A drop of the spore suspension ($5X10^5$ conidia/ml) of *A. solani* isolates prepared in sterile distilled water was placed in each cavity slide. The slides were incubated in a Petri dish glass bridge moist chamber. Spore suspension in sterile distilled water served as control. The spore germination was recorded after 48 h.

Correlation analysis

Using the mean values of mycelial growth and spore germination of different isolates of *A. solani*, correlation analysis was done with virulence index. Microsoft Excel software version 5.0 was used to determine correlation coefficients.

Results

The isolates of *A. solani* were designated as I_1 to I_{20} based on the virulence index *(Tables 1, 2)*. The isolate I_1 recorded 72.3 PDI on susceptible tomato cv. PKM 1 followed by I_2 (63.8 PDI). I_{20} was the least virulent with only 18.8 PDI. All other isolates showed no significant difference in virulence *(Table 1)*.

Table 1

Virulence index of *Alternaria solani* isolates on tomato plants

Isolate	Per cent Disease Index* (PDI)
I_1	72.3
I_2	63.8
I_3	52.4
I_4	50.0
I_5	47.3
I_6	46.6
I_7	46.0
I_8	44.0
I_9	40.9
I_{10}	39.3
I_{11}	36.0
I_{12}	35.3
I_{13}	53.3
I_{14}	32.7
I_{15}	28.2
I_{16}	26.7
I_{17}	22.9
I_{18}	22.2
I_{19}	20.0
I_{20}	18.9

*Mean of three replications
CD (p=0.05) 2.83

Table 2

Mycelial growth of *Alternaria solani* isolates in fungicide-amended culture media

Isolate	Mancozeb		Captofol		Copper oxychloride		Aureofungin		Chlorothalonil		Validamycin		Control (sterile water)
	Mycelial growth	Percent inhibition over control	Mycelial growth	Percent inhibition over control	Mycelial growth	Percent inhibition over control	Mycelial growth	Percent inhibition over control	Mycelial growth	Percent inhibition over control	Mycelial growth	Percent inhibition over control	
I_1	19.3	78.3	37.7	57.7	37.3	58.2	46.3	48.5	55.3	37.8	87.3	1.9	90.0
I_2	14.0	84.4	32.3	64.1	35.0	61.1	40.7	54.8	48.3	46.3	78.7	12.6	90.0
I_3	13.0	85.4	28.0	68.5	30.3	65.9	43.0	51.7	44.0	50.6	72.0	19.1	89.0
I_4	14.7	83.6	23.3	73.9	26.7	70.1	42.3	52.6	35.0	60.8	82.0	8.2	89.3
I_5	15.7	82.6	23.7	73.7	32.7	63.7	34.3	61.9	47.0	47.8	82.0	8.9	90.0
I_6	19.7	78.1	29.0	67.8	32.3	64.1	38.0	57.8	43.0	52.2	83.7	7.0	90.0
I_7	13.0	85.6	24.3	72.9	26.7	70.4	35.0	61.1	42.0	53.3	82.3	8.5	90.0
I_8	16.7	81.5	31.7	64.8	35.3	60.7	32.0	64.4	45.3	49.6	85.3	5.2	90.0
I_9	13.3	85.2	29.0	67.8	32.3	64.1	32.0	64.4	47.3	47.4	84.3	6.3	90.0
I_{10}	14.3	84.1	26.7	70.4	24.3	72.9	34.3	61.9	54.3	50.7	82.3	8.5	90.0
I_{11}	15.0	83.3	25.7	71.5	27.7	69.3	44.3	50.7	44.7	50.4	80.3	10.7	90.0
I_{12}	11.0	87.7	29.7	66.9	32.7	63.6	38.7	56.9	48.7	45.7	86.0	4.1	89.7
I_{13}	14.3	83.9	34.3	61.4	35.7	59.9	40.3	54.7	51.7	42.2	78.3	11.9	89.0
I_{14}	13.3	85.2	30.7	65.9	24.3	72.9	33.7	62.6	50.7	43.7	85.3	5.2	90.0
I_{15}	18.3	79.6	24.7	72.6	26.7	70.4	40.7	54.8	44.3	50.7	81.3	9.6	90.0
I_{16}	13.0	85.6	27.7	69.3	26.7	70.4	34.3	61.9	55.3	38.5	80.3	10.7	90.0
I_{17}	18.3	79.6	26.7	70.4	29.0	67.8	40.3	55.2	41.0	54.4	80.3	10.7	90.0
I_{18}	17.3	78.3	27.0	69.8	33.3	62.5	41.0	53.9	46.7	47.6	85.0	4.8	89.0
I_{19}	13.3	85.2	30.7	65.9	30.0	66.7	33.3	62.7	49.7	44.8	79.7	11.5	89.3
I_{20}	14.3	84.1	28.7	68.1	35.0	61.1	38.3	57.4	49.3	45.2	80.3	10.7	90.0
Mean	15.1		28.6		30.7		38.1		46.7		81.8		89.7

Mycelial growth in mm.

	CD (p=0.05)
Fungicides	0.69
Isolates	1.16
IsolatesXFungicides	3.09

Table 3

Spore germination of *Alteraria solani* isolates after fungicide treatment

Isolate	Mancozeb		Captofol		Copper oxychloride		Aureofungin		Chlorothalonil		Validamycin		Control (sterile water)
	Mycelial growth	Percent inhibition over control	Mycelial growth	Percent inhibition over control	Mycelial growth	Percent inhibition over control	Mycelial growth	Percent inhibition over control	Mycelial growth	Percent inhibition over control	Mycelial growth	Percent inhibition over control	
I_1	10.1	88.9	20.7	77.0	35.8	60.9	43.3	53.9	55.4	39.0	84.5	9.6	95.4
I_2	7.9	91.7	17.1	82.0	32.2	66.2	38.0	60.1	48.9	48.7	83.8	12.2	91.6
I_3	8.5	90.6	20.1	78.0	33.2	63.1	35.7	60.4	51.1	43.3	80.3	10.9	90.2
I_4	8.4	91.0	17.5	81.2	33.5	64.1	40.4	56.7	50.0	46.4	80.7	13.5	93.3
I_5	7.2	92.1	18.3	79.9	29.3	67.8	42.3	53.7	54.2	40.6	83.8	8.2	91.2
I_6	8.9	90.6	17.7	81.1	34.1	63.6	39.2	57.2	48.9	47.9	83.3	11.3	93.9
I_7	9.6	89.4	20.4	77.4	31.7	64.9	38.3	57.7	53.7	40.7	82.6	8.7	90.5
I_8	7.8	91.3	19.9	77.8	31.8	64.6	43.0	52.1	55.2	38.5	79.6	11.2	89.7
I_9	8.0	91.3	19.6	78.8	30.2	67.5	42.8	53.9	54.3	41.5	77.3	16.7	92.8
I_{10}	7.9	91.2	20.1	77.8	32.3	64.4	42.7	53.0	50.0	45.4	80.9	11.0	90.7
I_{11}	8.7	90.6	17.3	81.4	32.6	65.0	42.8	54.1	53.4	42.7	81.7	12.3	93.1
I_{12}	8.6	90.8	19.3	79.4	33.3	64.4	35.8	61.7	53.9	42.4	79.4	13.3	93.6
I_{13}	7.1	92.4	19.6	78.8	29.5	68.2	40.7	56.2	52.8	43.1	76.8	17.2	92.8
I_{14}	8.6	90.4	19.0	78.9	31.0	65.6	38.6	57.3	54.2	40.1	80.0	11.5	90.4
I_{15}	8.2	91.3	18.9	79.9	31.3	66.9	37.7	60.1	47.9	49.4	77.7	17.9	94.7
I_{16}	9.1	90.0	18.1	80.3	27.4	70.1	36.8	59.8	53.6	41.5	80.0	12.7	91.7
I_{17}	7.6	91.6	19.0	79.1	30.2	66.9	39.0	57.2	53.4	41.5	78.5	13.9	91.2
I_{18}	8.7	90.5	20.1	78.0	27.8	69.6	40.9	55.25	53.5	41.5	80.7	11.7	91.5
I_{19}	9.4	89.7	19.9	78.0	28.4	68.7	37.3	58.9	50.5	44.3	77.8	14.2	90.7
I_{20}	8.8	90.5	18.5	80.0	30.5	67.1	41.8	54.9	49.0	47.1	78.8	15.0	92.8
Mean	8.38		18.98		31.3		39.9		52.2		80.5		92.5

Mycelial growth in mm.

	CD (p=0.05)
Fungicides	0.87
Isolates	1.48
IsolatesXFungicides	3.91

All the fungicides tested significantly inhibited the mycelial growth and spore germination of *A. solani* isolates as compared to control. Among the fungicides, mancozeb (0.2%) was the most effective followed by captofol (0.2%). Validamycin D is least effective. Among the isolates, inhibition of mycelial growth by fungicides was the least in I_1, however maximum inhibition was observed in I_{12} in mancozeb, I_4 in captofol, I_{10} and I_{14} in copper oxychloride, I_8 and I_9 in aureofungin, I_4 in chlorothalonil and I_3 in validamycin *(Table 2)*.

Mancozeb was the most effective in inhibiting the spore germination also. Germination was highest in I_1 in all the fungicides *(Table 3)*. The per cent inhibition of spore germination was maximum in I_3 in mancozeb, I_2 in captofol, I_{16} in copper oxychloride, I_{12} in aureofungin and I_{15} in chlorothalonil.

Correlation existed between the virulence index and mycelial growth of *A. solani* isolates on fungicide amended medium except for chlorothalonil. The r values are 0.155, 0.159, 0.292, 0.316, –0.089 and 0.075 for mancozeb, captofol, copper oxychloride, aureofungin, chlorothalonil and validamycin, respectively. Similarly excluding for captofol (r = –0.016), correlation was detected between virulence index and spore germination after fungicide (r = 0.108, 0.717, 0.176, 0.100 and 0.709 for mancozeb, copper oxychloride, aureofungin, chlorothalonil and validamycin, respectively) treatment.

Discussion

Variability of plant pathogens and their optimum threshold levels are of great concern to workout strategies for the management of the disease (Krishnarao and Krishnappa, 1997). Preliminary investigations carried out revealed that the severity of leaf blight of tomato caused by *A. solani* varied from place to place in the same cropping season (Babu et al., 2000b). The fact that the isolates of *A. solani* differing in their virulence exist in different tomato growing localities as observed in the present study justifies the occurrence of leaf blight disease on tomato at varying intensities in these areas. The most virulent isolate is from the intensively cultivating area where the disease is a serious problem during cropping season. The least virulent isolate is from a place where the local less susceptible tomato variety is grown as compared to highly susceptible PKM 1 tomato popularly grown in the area from where virulent isolate was obtained. There are several reports on the variability in virulence of *Alternaria* sp. (Berry, 1959; Awasthi and Kolte, 1989; Koul and Saksena, 1989; Vishwanath and Kolte, 1997).

Mancozeb followed by captofol were found to be effective in reducing the mycelial growth and inhibiting the spore germination of *A. solani* isolates. These results are in agreement with the earlier reports by Choulwar et al. (1989). Inhibition of spore germination of *A. alternata* (Sharma et al., 1992) and *A. tenuissima* (Fazili, 1988) by mancozeb has also been reported. It is interesting to note that virulent isolate of *A. solani* is least inhibited by the fungicides and this trend is observed even with the effective fungicide.

Resistance to fungicides is now a serious issue in the management of crop diseases. Eventhough resistance has been restricted to systemic fungicides, few excep-

tions of broad-spectrum fungicides have also lead to development of resistance in the pathogen after constant and intensive use throughout many years (Hewitt, 1998). Gangawane (1997) reported the development of resistance to mancozeb in apple scab pathogen. Moreover, resistance builds up more rapidly in and abundantly sporulating pathogen on aerial parts of the plant than in slowly expanding soil pathogen (Hewitt, 1998). Acquired resistance in *Alternaria* to polyoxin has been recorded in apple (Eguchi et al., 1974) and pear (Shimada et al., 1973). Tolerance of some isolates of *A. brassicae* to fungicides was reported (Vishwanath and Kolte, 1997).

The *A. solani* isolates although not resistant to fungicides, are less sensitive as a positive correlation existed between the virulence and ability to grow and sporulate in the presence of fungicides. The present study has clearly indicated the existence of pathologically differing types of tomato leaf blight pathogen and some amount of tolerance has been achieved by the pathogen to the fungicides commonly used to control the disease. Further in-depth investigations based on baseline sensitivity assessments with different doses of fungicides would provide more information on the fungicide tolerance by tomato leaf blight pathogen.

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Role of a Host-specific Toxin Produced by *Rhizoctonia solani* in Suppression of Defense Mechanisms in Rice

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The effect of a host-specific toxin produced by *Rhizoctonia solani*, the rice sheath blight pathogen on the elicitor-induced accumulation of phenolics and activities of phenylalanine ammonia-lyase, chitinase and ß-1,3-glucanase in rice was studied. Treatment of rice leaf sheaths with elicitor isolated from the mycelial walls of *R. solani* induced the accumulation of phenolics and activities of phenylalanine ammonia-lyase, chitinase and ß-1,3-glucanase. Maximum increase was observed 48 h after treatment. The phenolic content and phenylalanine ammonia-lyase, chitinase and ß-1,3-glucanase activities were significantly reduced when rice leaf sheaths were treated with *R. solani* toxin. Pretreatment of rice leaf sheaths with toxin suppressed the elicitor-induced accumulation of phenols and activities of phenylalanine ammonia-lyase, chitinase and ß-1,3-glucanase.

Keywords: elicitor, *Oryza sativa*, pathogenesis-related proteins, phenols, phenylalanine ammonia-lyase, sheat blight.

Sheath blight, caused by the fungus *Rhizoctonia solani* Kuhn is one of the important diseases of rice (*Oryza sativa* L.) in India causing significant yield losses. Gene for sheath blight resistance has not been identified from the existing cultivars of rice (Bonmann et al., 1992). However, it is known that all higher plants, irrespective of whether they are resistant or susceptible, are equipped with a wide array of constitutive deterrents that include both toxic chemicals and mechanical barriers. In addition to these constitutive defenses, several defense responses are induced in plants in response to infection by pathogens. These defense responses are involved in either strengthening of barriers against invasion or in weakening and destroying of invading pathogens (Vidhyasekaran, 1997). These inducible defense responses include hypersensitive cell death (Levine et al., 1994), production of phytoalexins (Kuć and Rush, 1985), accumulation of pathogenesis-related (PR) proteins (Velazhahan et al., 1998; Velazhahan et al., 2000), induction of oxidative burst (Baker and Orlandi, 1995), lignification (Mitchell et al., 1994), cross-linking of wall glycoprotein (Bradley et al., 1992). The activation of these defense mechanisms is considered to be consequent upon an initial recognition event in which the plant detects molecular components of the pathogen, known as elicitors (Albersheim and Anderson-Prouty, 1975). Several elicitors have been isolated and characterized from fungal pathogens (for review, see Vidhyasekaran, 1997). We recently isolated and partially purified an elicitor from the mycelial walls of *R. solani* which activates phenylpropanoid metabolism (Velazhahan and Vidhyasekaran, 2000), antifungal chitinase (Velazhahan et al., 2000) and generation of active oxygen species (Velazhahan and Vidhyasekaran, 1999) in suspension-cultured rice cells.

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Successful pathogens, however, overcome the defense mechanisms of host and cause disease (Atkinson, 1993). The success of many pathogenic fungi depends upon their ability to detoxify or suppress the synthesis of defense chemicals (Shiraishi et al., 1978; Van Etten et al., 1989; Yamada et al., 1989). For example, the pathogens of tomato viz., *Fusarium oxysporum* f. sp. *lycopersici, Alternaria solani* and *Septoria lycopersici* are capable of detoxifying the tomato saponin α-tomatine (Arneson and Durbin, 1967; Ford et al., 1977). Similarly, a low molecular weight glycopeptide suppressor obtained from spore germination fluids of *Mycosphaerella pinodes* inhibits pisatin and accumulation of phenylalanine ammonia-lyase and chalcone synthase mRNAs (Yamada et al., 1989). Further, it has been demonstrated that certain strains of *Nectria haematococca* produce pisatin demethylase which convert pisatin into an inactive compound and confers insensitivity to the phytoalexin pisatin (Van Etten et al., 1989). Toxins produced by plant pathogens are known to suppress defense mechanisms of host plants (Patil, 1974; Hayami et al., 1980; Otani et al., 1980; Vidhyasekaran et al., 1992). The fungus, *Rhizoctonia solani* produces a host-specific toxin that reproduces all symptoms of the disease and induced electrolyte leakage from treated rice tissues (Vidhyasekaran et al., 1997). The purified toxin was found to be a carbohydrate containing glucose, mannose, N-acetyl-galactosamine and N-acetylglucosamine. In the present study we examined the effect of *R. solani* toxin in suppression of defense mechanisms in rice.

Materials and Methods

Isolation of elicitor and toxin

The fungus *Rhizoctonia solani* was isolated from the sheath blight infected rice (cv. IR50) plants and the culture was maintained on PDA slants. Elicitor was isolated from the mycelial walls of *Rhizoctonia solani* as described earlier (Velazhahan and Vidhyasekaran, 2000). A host-specific toxin was purified from the culture filtrate of *R. solani* by the procedure of Vidhyasekaran et al. (1997).

Effect of elicitor and toxin on defense mechanisms of rice

Leaf sheaths of 45-day-old rice plants (cv. IR50) were detached and cut in to 4 cm pieces. Each leaf sheath was placed on a glass slide and its ends were fixed with Scotch tape. An injury was made with the tip of a ballpoint pen. The slide was kept inside a Petri dish lined with wet blotting paper. Fifty µl of pretreatment solution viz., toxin (10 µg carbohydrate equivalents per ml) or elicitor (10 µg glucose equivalents per ml) or water was placed on a 4 mm diameter filter paper disc that was placed on the injured leaf sheath section and incubated for 6 h under laboratory conditions (25 ±2 °C). In the same place 50 µl of treatment solution (elicitor or toxin or water) was added and incubated further. At different time intervals, the samples were collected and various analyses were made.

Estimation of phenolic content

For estimating phenolic content, rice leaf sheaths (1 g) were homogenized in 10 ml of 80% methanol and agitated for 15 min at 70 °C (Swain and Hillis, 1959). One ml of the methanolic extract was added to 5 ml of distilled water and 250 µl of Folin-Ciocalteau reagent (1 N) and the solution was kept at 25 °C. After 3 min 1 ml of saturated solution of Na_2CO_3 and 1 ml of distilled water were added and the reaction mixture was incubated for 1 h at 25 °C. The absorption of the developed blue colour was measured using a Beckman DU64 spectrophotometer at 725 nm. The content of the total soluble phenols was calculated according to a standard curve obtained from a Folin-Ciocalteau reaction with phenol and expressed as phenol equivalents in µg g^{-1} fresh weight.

Determination of PAL activity

Rice leaf sheaths (1 g) were homogenized in 5 ml of 0.1 M sodium borate buffer, pH 7.0 containing 0.1 g insoluble polyvinylpolypyrrolidone (PVP). The homogenate was centrifuged at 10,000 rpm for 30 min. The supernatant was used in the enzyme assay. PAL activity was determined as the rate of conversion of L-phenylalanine to *trans*-cinnamic acid at 290 nm as described by Dickerson et al. (1984). Samples containing 0.4 ml of enzyme extract were incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30 °C (Dickerson et al., 1984). In reference cell, 0.4 ml of enzyme extract was taken along with 1.0 ml of borate buffer. The amount of *trans*-cinnamic acid synthesized was calculated using its extinction coefficient of 9630 M^{-1} cm^{-1} (Dickerson et al., 1984). Enzyme activity was expressed on a fresh weight basis (n mol min^{-1} g^{-1} fresh weight).

Assay of chitinase

Rice leaf sheaths (1 g) were homogenized in 5 ml of 0.1 M sodium citrate buffer (pH 5.0). The homogenate was centrifuged for 10 min at 10,000 rpm at 4 °C and the supernatant was used in the enzyme assay. Colloidal chitin was prepared according to Berger and Reynolds (1958) from crab shell chitin (Sigma). The commercial lyophilized snail gut enzyme (Helicase, obtained from Sepracor, France) was desalted as described by Boller and Mauch (1988). For the colorimetric assay of chitinase 10 µl of 1 M sodium acetate buffer (pH 4.0), 0.4 ml of enzyme extract and 0.1 ml colloidal chitin (1 mg) were pipetted into a 1.5 ml eppendorf tube. After 2 h at 37 °C, the reaction was stopped by centrifugation at 1,000 rpm for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 µl 1 M potassium phosphate buffer (pH 7.1) and incubated with 20 µl desalted snail gut enzyme for 1 h. The resulting monomeric N-acetylglucosamine (GlcNAc) was determined according to Reissig et al. (1955) using internal standards of GlcNAc in the assay mixtures for calculations. Enzyme activity was expressed as nmol GlcNAc equivalents min^{-1} g^{-1} fresh weight.

Assay of ß-1,3-glucanase activity

ß-1,3-glucanase activity was assayed by the laminarin-dinitrosalicylate method (Pan et al., 1991). Leaf sheaths were extracted with 5 ml of 0.05 M sodium acetate buffer (pH 5.0) by grinding at 4 °C using a pestle and mortar. The extract was then centrifuged at 10,000 rpm for 15 min at 4 °C and the supernatant was used in the enzyme assay. The reaction mixture consisted of 62.5 µl of 4% laminarin and 62.5 µl of enzyme extract. The reaction was carried out at 40 °C for 10 min. The reaction was then stopped by adding 375 µl of dinitrosalicylic reagent and heating for 5 min on a boiling water bath. The resulting coloured solution was diluted with 4.5 ml of distilled water, vortexed and its absorbance at 500 nm was determined. Enzyme activity was expressed as n mol min^{-1} g^{-1} fresh weight.

Results

Phenolic content

The phenolic content of rice leaf sheaths after treatment with elicitor started increasing at 12 h after treatment; the highest content was observed at 48 h after treatment *(Table 1). R. solani* toxin significantly decreased the phenolic content of rice leaf sheaths even at 12 h after treatment. Pretreatment of rice leaf sheaths with toxin suppressed the accumulation of phenols whether they were subsequently treated with elicitor or water. When the leaf sheaths were pretreated with elicitor, subsequent treatment with toxin had no effect on elicitor-induced accumulation of phenolics.

Table 1

Effect of *Rhizoctonia solani* toxin on elicitor-induced accumulation of phenolics in rice

Pretreatment 6 h	Treatment	Phenolic content (µg g^{-1} fresh weight)						
		0 h	12 h	24 h	36 h	48 h	60 h	72 h
Water	Water	511.7	516.6	520.3	523.3	516.7	510.3	515.0
Water	Elicitor	513.3	923.3	943.3	1021.7	1033.3	896.7	835.0
Water	Toxin	515.3	460.0	351.7	370.0	363.3	448.7	520.0
Elicitor	Toxin	683.3	851.7	913.3	960.0	966.7	863.3	773.3
Elicitor	Water	723.3	915.0	920.0	1026.7	1036.7	906.7	843.3
Toxin	Elicitor	471.6	436.7	420.0	376.7	356.7	443.3	533.3
Toxin	Water	463.3	473.3	376.7	353.3	371.7	408.3	520.0

Least significant difference to compare any two means at P = 0.05 is 56.0
The data are mean of 3 replications.

PAL activity

Treatment of rice leaf sheaths with elicitor significantly increased the activity of phenylalanine ammonia-lyase. Significant increase in PAL activity was observed at 24 h after treatment and the maximum activity was observed at 48 h after treatment *(Table 2).*

R. solani toxin significantly decreased PAL activity even at 12 h after treatment and the enzyme activity consistently decreased throughout the experimental period of 72 h. Pretreatment of rice leaf sheaths with toxin suppressed the elicitor-induced PAL activity. Rice leaf sheaths pretreated with toxin exhibited low PAL activity whether they were subsequently treated with elicitor or water. When the leaf sheaths were pretreated with elicitor, subsequent treatment with toxin had no effect on elicitor-induced activity of PAL.

Table 2

Effect of *Rhizoctonia solani* toxin on elicitor-induced activity of phenylalanine ammonia-lyase in rice

Pretreatment 6 h	Treatment	PAL activity (n mol min^{-1} g^{-1} fresh weight)						
		0 h	12 h	24 h	36 h	48 h	60 h	72 h
Water	Water	78.16	81.12	80.95	74.77	73.04	70.66	70.30
Water	Elicitor	76.31	84.00	92.01	107.58	124.60	117.10	85.95
Water	Toxin	75.39	63.74	49.68	45.62	34.10	26.67	20.54
Elicitor	Toxin	84.51	88.95	94.53	108.88	128.50	118.11	87.61
Elicitor	Water	85.16	91.58	100.30	110.76	130.23	123.71	85.85
Toxin	Elicitor	63.24	58.18	50.55	42.54	31.44	26.53	16.45
Toxin	Water	64.10	59.63	54.29	44.34	30.79	24.15	21.19

Least significant difference to compare any two means at P = 0.05 is 4.12
The data are mean of 3 replications.

Chitinase activity

Elicitor induced chitinase activity in rice leaf sheaths even at 12 h after treatment and maximum activity was detected at 48 h after treatment *(Table 3).* Toxin significantly suppressed the activity of chitinase even at 12 h after treatment when compared to control. Pretreatment of rice leaf sheaths with toxin significantly suppressed the elicitor-induced chitinase activity. When the leaf sheaths were pretreated with elicitor, subsequent treatment with the toxin had no effect on elicitor-induced activity of chitinase.

Table 3

Effect of *Rhizoctonia solani* toxin on elicitor-induced activity of chitinase in rice

Pretreatment 6 h	Treatment	Chitinase activity (n mol GlcNAc min^{-1} g^{-1} fresh weight)						
		0 h	12 h	24 h	36 h	48 h	60 h	72 h
Water	Water	12.12	12.24	11.76	12.32	11.76	12.36	12.13
Water	Elicitor	11.78	17.33	25.87	31.90	37.31	28.51	24.80
Water	Toxin	11.76	9.73	8.66	6.47	5.71	4.27	2.62
Elicitor	Toxin	13.35	18.93	26.52	34.36	38.96	28.45	25.02
Elicitor	Water	11.97	17.69	23.36	32.74	38.62	27.65	26.70
Toxin	Elicitor	10.01	8.76	7.28	6.02	4.27	3.38	2.64
Toxin	Water	10.36	7.78	5.48	3.70	3.24	2.69	2.29

Least significant difference to compare any two means at P = 0.05 is 1.58
The data are mean of 3 replications.

ß-1,3-glucanase activity

Treatment with the elicitor significantly enhanced the activity of ß-1,3-glucanase in rice leaf sheaths. Significant increase was observed at 12 h after elicitor treatment and the highest glucanase activity was observed at 36 h after treatment *(Table 4)*. Subsequently the enzyme activity declined but remained significantly higher level than the control. Treatment of rice leaf sheath with toxin significantly suppressed the glucanase activity even at 24 h after treatment. When the leaf sheaths were pretreated with toxin, activity of the elicitor in inducing ß-1,3-glucanase was significantly suppressed.

Table 4

Effect of *Rhizoctonia solani* toxin on elicitor-induced activity of ß-1,3-glucanase in rice

Pretreatment 6 h	Treatment	ß-1,3-glucanase activity (nmol min^{-1} g^{-1} fresh weight)						
		0 h	12 h	24 h	36 h	48 h	60 h	72 h
Water	Water	311.6	311.7	329.9	311.2	307.4	303.1	311.2
Water	Elicitor	303.1	593.3	862.3	1225.9	1199.5	939.1	776.9
Water	Toxin	329.9	298.9	243.3	183.6	166.3	140.7	183.6
Elicitor	Toxin	345.8	502.3	879.4	1250.8	1178.2	960.5	755.6
Elicitor	Water	345.8	618.9	871.9	1167.3	1131.2	900.7	738.5
Toxin	Elicitor	282.7	234.8	196.4	175.0	145.1	175.0	200.6
Toxin	Water	275.8	237.4	196.6	183.3	131.8	186.5	204.2

Least significant difference to compare any two means at P = 0.05 is 60.6
The data are mean of 3 replications.

Discussion

Several studies suggest that toxin may suppress defense mechanisms of plants (Tamari et al., 1965; Patil, 1974; Otani et al., 1975; Kohmoto et al., 1976; Gnanamanickam and Patil, 1977; Hayami et al., 1980; Tietjen and Matern, 1984; Vidhyasekaran et al., 1992; Fujita et al., 1994). Mayama et al. (1986) reported that treatment of oats susceptible to *Cochliobolus victoriae* with high concentration of victorin greatly reduced the accumulation of avenalumin. Vidhyasekaran et al. (1992) reported that *Helminthosporium oryzae* toxin caused a decrease in phenolic content and the activity of PAL in rice. The host-specific toxin released by certain fungal pathogens acts as a host recognition factor during spore germination at the infection site in plants. Prior to penetration of the pathogen into its host, the released toxin specifically binds to a putative receptor on the host cells and initiates signaling mechanisms leading to immobilization of the host's defense reactions and predispose the cells to being in a "susceptible state" for fungal penetration (Kohmoto and Otani, 1991). The existence of toxin receptors has been demonstrated in the interactions between victorin produced by *Cochliobolus victoriae* and oats. Electrophoretic analysis of leaf proteins from isogenic susceptible and resistant oat genotypes after treatment with the ^{125}I-labeled victorin derivative indicated that the toxin binds in a covalent and genotype-specific manner to a 100-kDa protein only in susceptible leaves (Wolpert et

al., 1988) which may act as toxin receptor. In the present study, pretreatment of rice sheaths with *R. solani* toxin suppressed the elicitor-induced activities of chitinase, glucanase, PAL and accumulation of phenolics. PAL is the key enzyme of phenylpropanoid metabolism in higher plants which leads to synthesis of phenols (Massala et al., 1980). Increase in phenolic content in plants has been correlated with enhanced resistance to pathogens (Velazhahan and Vidhyasekaran, 1994). Since the production of phenolic compounds depends upon PAL activity (Graham and Graham, 1991), decreased phenolic synthesis in toxin treated rice tissues may be due to decreased activity of PAL.

Several studies have shown that PR-proteins are induced in plants upon inoculation with pathogen or treatment with elicitors (Velazhahan et al., 1998; Velazhahan et al., 2000). Accumulation of PR-proteins is associated with systemic acquired resistance (SAR) in plants (Ryals et al., 1996). PR-proteins like chitinase and ß-1,3-glucanase have the potential to hydrolyze chitin and ß-1,3-glucan, respectively, which are major components of fungal cell walls. Moreover the chitinase and glucanase release the elicitors from the walls of fungi which, in turn, stimulate various defense responses in plants (Ham et al., 1991; Ren and West, 1992). Furthermore, it has been demonstrated that overexpression of chitinase and glucanse in transgenic plants was associated with enhanced resistance to various fungal pathogens (Broglie et al., 1991; Zhu et al., 1994; Lin et al., 1995). In the present study it was observed that elicitor induced the activities of chitinase and glucanase. However, pretreatment of rice tissues with *R. solani* toxin suppressed the elicitor-induced activities of chitinase and glucanase.

The recognition of fungal elicitors by receptors at the host plasma membrane is considered as the most important events leading to induction of defense mechanisms (Dixon and Lamb, 1990). Activation of plant defense responses by fungal elicitors appears to involve protein phosphorylation (Felix et al., 1994; Viard et al., 1994). Zhang et al. (1998) reported that a cell wall-derived carbohydrate elicitor and two proteinaceous elicitins, parasiticein and cryptogein, from *Phytophthora* spp. activated a 48-kDa kinase in tobacco cells which was identical to the salicylic acid-induced protein kinase (SIP kinase). They further demonstrated that inhibition of SIP kinase by kinase inhibitors correlated with suppression of PAL gene activation induced by the fungal elicitor. They concluded that the SIP kinase might participate in the activation of the elicitor-induced defense responses in tobacco. *R. solani* toxin is known to induce electrolyte leakage in rice tissues (Vidhyasekaran et al., 1997). The target site for many host-specific toxins is plasma membrane (Kohmoto and Otani, 1991). The dysfunction in plasma membrane of rice cells due to *R. solani* toxin might have suppressed the elicitor-induced increase in accumulation of phenolics and activities of chitinase and glucanase. However, Ransom et al. (1992) reported that synthesis and accumulation of 3-deoxyanthocyanidin phytoalexins in root tissues of the susceptible, toxin-sensitive genotype of sorghum were not suppressed when inoculated with *Periconia circinata,* which produces PC-toxin during pathogenesis.

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Walnut Pathogens in Hungary

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Two-year-study has been made to establish the bacterial and fungal pathogens of walnut trees in Hungary. *Phomopsis, Diplodia, Cytospora* and *Phoma* were the most important species infecting the trees. *Xanthomonas* and *Gnomonia* influenced the quality of walnut, while mould fungi infected the stored walnuts. This findings help to improve the technology of plant protection.

Keywords: walnut pathogens, *Phomopsis, Diplodia, Cytospora, Phoma, Gnomonia, Xanthomonas.*

Walnut (*Juglans regia* L.) is one of our most valuable tree species in Hungary. It is produced primarily for its shelled nuts. In addition, there is a big demand for walnut wood in the timber industry (Szentiványi, 1958).

In the past years there were no satisfactory amounts in yield of walnut and the quality was also not sufficient in spite of several (7–11 times) sprays. Amount and quality of harvested fruit and felled timber were reduced by not only to direct crop damage (bad fertility, fallen fruits, moulding), but by foliage damage and the death of branches, too. According to the experts the walnut will be an article much called for.

In the last decade a lot of publications were issued about the diseases and pathogens of walnut. In Hungary there was only one important virus and one bacterium species were mentioned: the cherry leaf roll virus and *Xanthomonas campestris* pv. *juglandis* bacterium species (V. Németh et al., 1982; Folk and Glits, 1993).

However, numerous pathogen fungi damaged the walnut trees. *Phytophthora* species of *Oomycota* played an important role (Farr et al., 1989), and among the *Ascomycota*-fungi the occurrence of about 30 species were wellknown. Among them most of important was *Gnomonia leptostyla* (Balaz et al., 1991; Véghelyi, 1994, 1997, 1998; Woeste and McGranahan, 1992). Basidiomycota-fungi – approximately 8–10 species – mainly cause the decay of walnut trees (Vajna, 1983; Sárvári, 1996).

Finally the *Deuteromycota* fungi come also up to 30 species. *Marssoniella juglandis, Phomopsis mali, Phoma juglandina, Diplodia juglandis, Cytospora* spp., *Sphaeropsis* spp. occurred frequently (Belisario, 1992, 1996; Pintér et al., 1999). The number of walnut pathogenic fungi is significantly more than it was thought earlier (Kadlicskó et al., 2000).

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Materials and Methods

Since 1998 summer surveys have been made weekly and monthly, respectively, in walnut orchards. Leaf samples, flowers, fruits, shoots and branches were collected from trees showing diseases symptoms. During two springs samples were collected from leaf-litter, too. The samples were examined with stereo- and binocular microscope in laboratory. A part of the samples was put into the wet-chamber, and the incubation was followed by microscope test or cultivation on culture-medium, then individual pathogen genus and species were identified.

Pathogenicity tests has been made on the host plant with the part of the isolated and identified bacterium and fungi i.e. reinoculations were carried out.

Results and Discussion

Leaf and fruit pathogens caused quantity and quality damages both directly and indirectly. The most important fungal species were identified *(Table 1)*. The following fungi species were also frequent on the dried couples of fruit or on the ones that were dried on the shell: *Epicoccum nigrum, Alternaria alternata, Periconia* sp.

Table 1

The most important pathogen fungal species isolated from leaves and fruits of walnut trees

Fungal species		Symptoms	Disease severity*	Notes
Teleomorpha	Anamorpha			
Gnomonia leptostyla	*Marssoniella juglandis*	circular, oviform spots on leaves, green fruits and shoots	+++	most frequent walnut disease
–	*Microstroma juglandis*	yellow spots on adaxial surface of leaves, white mould on abaxial surface	+	rare disease
–	*Alternaria* sp.	infection of fruit wall and fruit	+	rare disease
Diaporthe perniciosa	*Phomopsis mali*	early and late infection of fruit, brown colour on shelled walnuts	+++	frequent
–	*Trichothecium roseum*	pinkish mould on fruit wall, and on leaves	+	frequent
–	*Fusarium* sp.	infection of fruit, moulding	++	relatively frequent
–	*Aspergillus* sp.	infection of fruit, moulding	+	may occur

*+++ serious; ++ moderately serious; + not serious

Alternaria sp., *Penicillium* sp., *Aspergillus* sp. and *Mucor* sp. were found most frequently on diseased, raincid and discoloured shelled nuts, but *Fusarium avenaceum* and *F. semitectum* caused moulding occasionally too. The ruined-shelled nuts had bad taste and also contained mycotoxin. According to our two-year-study different fungi *(Table 2)* ruined the wooden parts of the walnut.

Table 2

More important pathogenic fungal species isolated from branches

Fungal species		Symptoms	Disease severity*	Notes
Teleomorpha	Anamorpha			
Diaporthe perniciosa	*Phomopsis mali*	dark necrosis and branch withering	+++	frequent
–	*Phoma juglandina*	frequent on dry branches, ulcerous bark	+++	frequent
–	*Camarosporium juglandis*	on part of necrotized branch	++	may occur
Gibberella avenacea	*Fusarium avenaceum, F. semitectum*	on twig, part of branch from dead tissues	+	weakening wound-parasites
–	*Diplodia juglandis*	on dried branches	++	may occur
–	*Microdiplodia* sp.	on dried branches	++	may occur
–	*Cytospora juglandina*	necrotized branches	++	may occur
Melanconis carthusiana	*Melanconium juglandinum*	bark necrosis on branches	+	frequent
Nectria cinnabarina	*Tubercularia vulgaris*	reddish warlike stroma on dried branches	+	weakening wound-parasite

*+++ serious; ++ moderately serious; + not serious

There were very frequent occurrences of shoot-end decay and fruit-stalk mortality on several walnut varieties. The rate of pathogens isolated from difference plant parts of walnut trees were sum up in *Table 3.* Infections caused by *Phomopsis mali* and *Phoma juglandina* were the most frequent. In some cases there was not possible isolate fungal pathogens from the examined plant parts. Mutual effects of more abiotic factors were supposed besides fungal infections.

In the pathogenicity tests made by *Phomopsis mali* the margins of the wounds became red-brown coloured, while the *Phoma juglandina* infections this discoloration did not develop. In the wake of infections the part of bark necrotised. After the *Phomopsis* infection big mass of picnidia developed in the bark tissues and after their swelling conidia mass rosee burst to the surface as protruding cirriform. Both conidia types (α and ß conidia) were frequently occurred. Following the *Phoma* infection developed on the bark parts, the hadrome became distinctly visible. Picnidia developed on margins of hadromes and bark tissues.

Table 3

Shoot-end decay and fruit-stalk infection on walnut trees
Lengyeltóti 2000. (March – April month)

Examined plant part	Occurrence of isolated fungi						Total number of samples
	Phomopsis mali %	*Phoma juglandina* %	*Diplodia juglandis* %	*Micro-diplodia* sp. %	*Melanconis carthusiana* %	No fungal infection %	336
Fruit-stalk	50.81	12.43	2.16	0.54	17.83	16.23	185
Fruit-epicarp	33.82	10.29	1.47	0.0	13.23	41.19	68
Shoot-end browning	21.53	6.15	6.15	1.53	4.61	60.03	65
2-year-old twig	57.89	0.00	15.78	0.00	0.00	26.33	19

On the green sprouts infected by *Marssoniella juglandis* after ripening, superficial bark necrosis developed in the following year. This fungus overwintered not only in teleomorf form (as *Gnomonia leptostyla*) but also in anamorf form on the twigs (as *Marssoniella juglandis*), and conidia could execute primary infections, too.

Additionally, other timber putrefactive fungi also appeared *(Trametes, Armillaria, Schizophyllum)* on thicker branches and trunks. The identified fungal species could cause early death of twigs and branches. Timber putrefactive by *Basidiomycota*-fungi caused brown decay, the blackening of walnut stumpwood and quality damage.

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Effect of Benzimidazole on Compatibility in the Wheat–Leaf Rust System

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Leaf segments, placed on benzimidazole solution were used to evaluate wheat samples for resistance to leaf rust. Most collection samples and somaclonal variants with low types of reaction at benzimidazole concentration 60 ppm were susceptible to the disease at lower concentrations (0, 15, 30 ppm). Isogenic lines of cv. Thatcher with non-effective juvenile genes Lr for resistance were susceptible at 60 ppm concentration, but lines with genes Lr 3bg, 13, 30 were resistant at 80 ppm concentration of benzimidazole and lines with genes Lr 2a, 15, 16, 18, 26, 23 differentially interacted with pathogen clones. Results of resistance evaluation in somaclones, obtained from one variety, and in isogenic lines showed, that type of reaction changes at different concentrations of benzimidazole is connected with effect of the chemical to gene(s) for resistance expression and not to overall plant genotype. If this assumption is correct, evaluation of wheat resistance to leaf rust at different concentrations of benzimidazole may be useful determining resistance.

Keywords: wheat, leaf rust, qualitative resistance, gene for resistance.

The use of "benzimidazole" method (Mikhailova and Kvitko, 1970) in assessment of plant resistance to harmful organisms has many benefits. It is time and place consuming, gives unique possibility to study reaction of single plant in segregating population to different isolates of pathogen or under different conditions. It is widely used to study interaction of plants with obligate parasites, including *Puccinia recondita* Rob. ex Desm. (Tyryshkin and Mikhailova, 1989) and facultative ones (Hetzler, 1992; Tyryshkin and Voronkova, 1997). The preferable concentration of the chemical in wheat researches is 60 ppm (60 mg/l) because in this case leaf segments do not loose their vitality during more than 10 days (Mikhailova and Kvitko, 1970). Higher concentrations are not desirable because of evident fungicide action of benzimidazole (Hetzler, 1992). Naturally it is supposed the same level of resistance in intact plants and leaf segments placed on water solution of benzimidazole, especially in case of qualitative resistance (Tyryshkin and Mikhailova, 1989). In our preliminary study of resistance to leaf rust in wheat somaclones (Tyryshkin, 1997) we found different types of reaction in leaf segments of the same plants at different concentration of benzimidazole. The general aim of the work was to study the possible effect of benzimidazole concentration to compatibility in wheat–leaf rust interaction.

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Materials and Methods

In the stage of first leaf 571 samples of winter wheat from All-Russian Institute of Plant Industry collection, 408 collection samples of spring wheat and 24 lines of somaclonal variants of variety Spica were studied for reaction to leaf rust with use of "benzimidazole" method. All collection samples are varieties or lines of modern breeding with different origin. Somaclones were selected during 8 generations for stable seedling (assessed with "benzimidazole" method, concentration of the chemical 60 ppm) and adult resistance to leaf rust (Tyryshkin, 1997). Lines with the same first number are progeny of one regenerated plant. Variety Vera was used as susceptible control in all experiments.

Segments of leaves (1–1.5 cm in length) were placed on cotton wool wetted with water solution of benzimidazole (60 ppm); water suspension of uredospores of *P. recondita* population was sprayed on them, cuvettes with leaves were covered with glasses and placed under light with temperature 20–22 °C. Type of reaction was scored after 7–8 days according to Mains and Jackson (1926). Samples with types 0–2 were considered resistant, that with types 3–4 susceptible. Population of the pathogen was collected from naturally infected wheat in St. Petersburg region. It is avirulent on lines with genes Lr 9, 19, 24, and virulent to genes Lr 1, 2a, 2b, 3bg, 3ka, 12, 13, 14a, 14b, 15, 16, 17, 18, 21, 23, 26, 30.

All genotypes with resistant reaction were studied for resistance with the same method, but with use of different concentration of benzimidazole – 0, 15, 30, 60 ppm.

Additionally isogenic lines of variety Thatcher with known genes for leaf rust resistance were assessed for resistance to leaf rust population with the same method at benzimidazole concentration – 0, 15, 30, 60, 80, 100 ppm.

Results

Among 979 collection samples under study 54 was resistant to *P. recondita* population used in the experiment at benzimidazole concentration 60 ppm *(Table 1);* most of them had types of reaction 0, 0; and 1. Only 15 samples were classified as resistant when their leaves were placed on cotton wool wetted with water (benzimidazole concentration 0). We did not observe any case when type of reaction was higher at higher concentration of benzimidazole. According to data all selected genotypes can be divided into 4 groups: 15 were resistant at all concentrations, 25 only at concentration of benzimidazole 60 ppm, 8 – at 15, 30 and 60 ppm and 6 – at 30 and 60 ppm *(Table 1).*

All 24 lines of somaclonal variants were highly resistant to leaf rust when benzimidazole concentration was 60 ppm, but only 8 of them had low types of reaction when leaf segments placed on cotton wool wetted with water were used for inoculation *(Table 2).* On the same leaves of many lines at benzimidazole concentrations 15 and 30 ppm different types of reaction were observed. Depending on lines these types represented resistant reaction or resistant and susceptible ones.

Table 1

Reaction of leaf segments of wheat collection samples, placed on different concentration of benzimidazole solution, to infection with population of leaf rust causal agent

Samples	Reaction at benzimidazole concentration (ppm)			
	0	15	30	60
Admiral, Alina, Arapahoe, Coda, Conveyor, Feng Kang 2, Hammer, Heinrich, MN 81330, ND 586, Siouxland 89, Tam 200, Tules, Wampum, Xing Mai 9	R*	R	R	R
Century, Ne 83498, Ne 87612, Rawhide 3, Sarno, Tam 201, Volnitsa, Weston	S	R	R	R
CRL 81025, Erithrospermum 14, Haven, Koda, Mildress, TAM 108	S	S	R	R
Belotserkovskaja 7, Bijon, Bu 27, Cabson de Joni, Capelle-Desprez, Carmen, Cherokee, Clement, Damier, Feng Kang 8, Flamingo, Grebe, Helios, Kentana 52, Korsodor, KS 8018-7-2, Phoenix, Roxana, Ruband, SO 137-78, Tjelvar, Unic, Vinkovacka, Vladimirskaja 98, Wong Da 84015	S	S	S	R

*R – resistant types of reaction; S – susceptible ones

All isogenic lines of Thatcher were resistant to leaf rust at benzimidazole concentration 100 ppm, but under this condition susceptible control had type of reaction 0, 1, 2 (resistance). Lines with effective genes Lr 9, 19, 24 always were resistant, 9 lines with non-effective genes were always susceptible *(Table 3)*. Lines with genes Lr 3bg, 13, 30 had low types of reaction at highest benzimidazole concentration used (80 ppm). At this concentration on the leaves of lines with Lr genes 2a, 15, 16, 18, 26, 23 only rare single pustules were formed.

Discussion

Use of "benzimidazole" method for plant resistance evaluation is based on the assumption, that types of reaction of intact plant and excised leaves do not differ. Concentration of the chemical was supposed not to influence type of reaction and was chosen to keep leaves alive for time that is necessary for disease development.

In our work the dependence of type of reaction to infection with leaf rust causal pathogen on benzimidazole concentration was found for collection wheat samples with unknown genetics of resistance, somaclonal variants, previously selected for leaf rust resistance, possibly controlled by polygenes (Tyryshkin et al., 2000) and lines of Thatcher with monogenic control of the trait.

Less than half of collection samples selected as resistant to leaf rust at concentration of benzimidazole 60 ppm were resistant when leaves were placed on water (15

from 54), some samples were susceptible at 15 and 30 ppm concentrations *(Table 1).* Obviously, to select "the best" concentration for practical purposes resistance of intact plants should be studied.

All 24 somaclonal lines were resistant to leaf rust at concentration of benzimidazole 60 ppm *(Table 2);* it was expected because during 7 generations selection for this trait was performed. At lower concentrations only 8 lines were uniformly resistant. At concentration of benzimidazole 15 and 30 ppm different types of reaction were observed on the same leaves of 21 lines; it may be explained by difference between isolates of the pathogen in degree of compatibility with the line or "occasional" development of symptoms due to small differences in concentration of benzimidazole.

Table 2

Type of reaction of somaclonal variants leaf segments, placed on different benzimidazole concentrations, to infection with leaf rust causal agent

Family R_6 of somaclonal variants	Number of R_8 lines	Type of reaction at benzimidazole concentration (ppm)			
		0	15	30	60
956, 3, 2	4	1, 2	0;, 2	0;, 1	0;
956, 3, 2	1	3	2, 3	0;, 1, 2, 3	0;
956, 3, 2	1	0, 3	1, 4	0; 1	0;
937, 6, 6	2	3	2	1, 2	0;, 1
937, 6, 6	2	1	1, 2	0; 1, 2	0;
937, 5, 2	3	1, 2, 3	1, 2, 3	0;, 1, 2	0;
937, 5, 2	1	3	1, 2, 3	0;, 1, 2, 3	0;
937, 9, 8	3	3	0;, 1, 2, 3	1, 2, 3	0;
937, 9, 8	4	2, 3	1, 2, 3	0;, 1, 2	0;
942, 6, 1	1	0	0	0;	0;
942, 13, 1	1	2	1	0;	0;
942, 13, 1	1	2	2	1	0

Most near-isogenic lines of Thatcher had the same characteristics for resistance at different concentration of benzimidazole: lines with effective genes Lr 9, 19, 24 were resistant, with non-effective ones susceptible. Lines with non effective genes Lr 3bg, 13, 30 were resistant to rust at unusual high concentration of benzimidazole 80 ppm; lines with genes 2a, 15, 16, 18, 26, 23 at this concentration differentially interacted with the pathogen isolates.

Evident dependence of type of reaction (index of qualitative resistance) to infection with *P. recondita* on concentration of benzimidazole was showed for 39 collection wheat samples *(Table 1).* This may be explained by effect of the chemical to expression of gene(s) for resistance or to action of another genes or overall genotype. Different mode of benzimidazole concentration influence on type of reaction in somaclonal variants of one

variety and even in progeny of one regenerated plant, as well as in near isogenic lines with different genes for resistance *(Tables 2, 3)* indicates that first hypothesis is more probable. In this case, in addition to hybridological analysis and phytopathological test analysis of resistance at different concentration of benzimidazole can be useful for preliminary dividing of genotypes under study into groups for further genetic researches.

Table 3

Type of reaction of leaf segments of isogenic Thatcher lines, placed on different benzimidazole concentration, to infection with leaf rust causal agent

Line with Lr gene	Type of reaction at benzimidazole concentration (ppm)				
	0	15	30	60	80
1, 2b, 3ka, 12, 14a, 14b, 17, 21, Ech	3	3	3	3	3
2a, 15, 16, 18, 26, 33	3	3	3	3	3 s.p.*
9	0	0	0	0	0
3bg, 13, 30	3	3	3	3	0
19	0; 2	0, 1	0	0	0
23	3	3	3	1, 2, 3	0, 1, 2
24	1	0, 1	0; 1	0; 1	0

*single pustules

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Resistance to Stem Rust (*Puccinia graminis* Pers. f. sp. *secalis* Erikss. et Henn.) in Rye

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Development of stem rust in short-stem diploid rye decreased grain yield by 35%. Breeding of resistant varieties is the most effective way of protection from the disease. Plants resistant to stem rust were selected from populations of diploid winter rye varieties received from the collection of our institute such as: Ilmen, Orlovskii Gibrid, Kharkovskaya 60, Kharkovskaya 55, Kustovka, Kombaininyai, Kazanskaya, Krupnozernaya, Novozybkovskaya 4, Alfa, Derzhavinskaya 29, Chulpan, Rossul and in perennial rye Secale montanum Guss. Genetic control of resistance was determined for Kharkovskaya 55 and Rossul. In both cases one single dominant gene resistance was found; we suppose that their genes for resistance were identical. We suggest a temporary symbol Sr1 for this gene.

Keywords: rye, stem rust resistance, genetic control.

Stem rust of rye (caused by fungi *Puccinia graminis* Pers. f. sp. *secalis* Erikss. et Henn.) is widespread disease in Russia. Wide distribution of the disease is attributed to wide specialization of forma special secalis. In scientific articles 18 plants genera were described, that can be naturally infected with rye stem rust. Main sources of infection are *Agropyrum* species: *A. repens* (L). Beauv., *A. elongatum* (Host) Beauv., *A. intermedium* (Host) Beauv. Uredospores of the causal pathogen, formed on plants of *Agropyrum,* can successfully infect rye plants and, vice versa, the inoculum from rye, easily infects all species of *Agropyrum* (Trushko, 1974). Trushko (1973) considered that rye is one of the sources of the infection for spring wheat in Western Siberia. In experiments of Sorokina (1991), it was shown that wheat stem rust biotypes could infect rye.

Heavy development of the stem rust can decrease grain yield up to 50–60% in rye (Shestiperova, 1962; Peresypkin, 1981). In the years of epiphytoty complete yield losses was stated (Chumakov, 1966; Chumakov and Sidorenko, 1973; Trushko, 1974; Suzdalskaya et al., 1974; Kucmierz and Gondek, 1975; Fochenkova et al., 1991). In investigations of Shaduro (1992) development of complex of diseases (leaf and stem rust) on short-stem winter cultivars of diploid rye resulted in decrease of 1000 grains weight by 50.4–52.7%.

First studies of genetic control of stem rust resistance in rye were conducted in 1926 (Mains, 1926). Author showed dominant inheritance of the trait. Until recently in the literature there was only information about dominance of resistance over susceptibility (Kobyliansky, 1975).

Some authors described presence of resistant plants in populations of several rye varieties (Koroleva, 1978; Shaduro, 1992). It created the possibility of rye breeding for stem rust resistance (Kobyliansky and Koroleva, 1977; Kobyliansky and Solodukhina, 1987).

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Negative influence of stem rust to the efficiency of rye cultivation, absence of resistant varieties, fragmentarty of studies of resistance inheritance have resulted in necessity of studies on genetic bases of plant resistance with reference to the aims of breeding, and also of the disease effect concretisation.

Materials and Methods

In our experiments rye plants were selected from varieties and forms (VIR collection) with resistance to the pathogen populations from Moscow and St. Petersburg regions. Resistant plants were identified with various frequency in varieties: Ilmen, Orlovskiy Gibrid, Kharkovskaya 60, 55, Kustovka, Kombaininaiy, Kazanskaya, Krupnozernaya, Novozybkovskaya 4, Alfa, Derzhavinskaya 29, Chulpan, Rossul and in perennial rye Secale montanum.

Genetic control of resistance was determined with analysis of backcrosses (BC_4) from crosses of resistant plants (R) (type of reaction 0) and susceptible (S) (type of reaction 3–4 according to the scale Mains and Jackson, 1926) ones from Getera 2 and Hja-6910 and F_2 from cross-pollination of heterozygous plants.

To study damage from the disease, F_2 plants from crosses between resistant and susceptible short-stem plants, both selected from variety Getera 3 were used. Experiment was conducted on artificially infected plants at field conditions. Plants resistant and infected in different degree (according to the scale R. F. Peterson et al., 1948) were compared. We estimated weight of 1000 grains, as a main metric index of damage from rust diseases (Simons, 1966).

Results and Discussion

In our work genetic control of resistance was determined for resistant plants from Kharkovskaya 55 and Rossul *(Table 1)*. The ratio of resistant to susceptible plants in BC_4 corresponded to theoretically expected 1:1 ($\chi^2 = 0.02–0.73$), and in F_2 – to the ratio 3:1 ($\chi^2 = 0.06–1.04$). This result indicated that stem rust resistance in resistant plants of Kharkovskaya 55 and Rossul is controlled by dominant allele of a single gene. Genealogical studies of these varieties showed that they have genetic relationship in their origin, as in their breeding the same donors of valuable traits were used. Outgoing from this, it is possible to suppose that dominant genes for resistance in Kharkovskaya 55 and Rossul are identical.

Australian scientists Tan et al. (1975; 1976; 1977) studied genetic control of stem rust resistance in rye. The study of stem rust resistance was carried out according to tasks of wheat, but not rye, breeding and the main purpose of those researches was an introgression of rye resistance to wheat genotypes. As a result of screening of the 60 self-compatible rye lines they selected 4 lines created on the basis of varieties Gotor 28, Elbon, Kenya 12, Wrens 5. Four lines were resistant to two races of wheat stem rust with different

Table 1

Segregation for stem rust resistance in BC_4 and F_2 from crosses of resistant rye plants with susceptible ones (1988–1989)

The combination of crossing	Generation of hybrids	Ratio resistant:susceptible		χ^2
		observed	expected	
(Kharkovskaya 55 HL RPg x 4 Getera 2)	BC_4	30:32	1:1	0.06
	BC_4	26:27	1:1	0.02
	BC_4	47:50	1:1	0.09
	BC_4	48:40	1:1	0.73
(4 Rossul RPg x Hja-6910)	BC_4	171:168	1:1	0.03
	F_2	159:55	3:1	0.06
	F_2	106:36	3:1	0.01
	F_2	74:19	3:1	1.04

virulence phenotype and to 1 clone of rye stem rust. As a result of this investigation 9 genes (SrA, SrC, SrD, SrE, SrJ, SrF, SrL, SrM, SrN) were postulated, among them 6 were possibly unlinked. The different genes express at different phases of plant development, control different types of resistance and differ in degree of recessiveness or dominance.

As opposite to letter symbols, proposed by Tan et al. (1976, 1977) for rye genes, controlling resistance to two biotypes of wheat stem rust and to one clone of rye stem rust, we suggested a temporary symbol Sr1 for dominant gene, controlling resistance of varieties Kharkovskaya 55 and Rossul to rye stem rust population.

Additionally the effect of stem rust to yield losses was determined for short-stem winter cultivars of diploid rye. It is known, that short-stem rye plants are more severely damaged by diseases than long-stem varieties (Kobyliansky, 1982).

The segregation of hybrids F_2 from crosses between resistant and susceptible short-stem plants of variety Getera 3 has allowed us to select and compare a group of resistant plants, infected by stem rust in range 0–10%, and susceptible ones, infected by stem rust in range 20–40%, 50–60%, and 70–100%. Plants with 0–10% of injured leaf area were used as control. In the population of Getera 3 there are plants resistant to complex of diseases: leaf rust, powdery mildew, fusarium stem rots. Using such plants in experiment for studying damage from stem rust, has allowed us to eliminate the influence of leaf rust, powdery mildew, fusarium stem rots to the development of grain yield. Thus, the differences in productivity of resistant and susceptible plants are mainly connected only with their differences in degree of stem rust infection. Our experiments have shown the dependence of weight of 1000 grains decrease on the degree of stem rust development *(Table 2, Fig. 1)*. Maximum development of the disease (70–100%) resulted in the weight of 1000 grains decrease by 35.8 percents.

Table 2

Loss of weight of 1000 grains in short-stem plants Getera 3 with different degree of stem rust infection (St. Petersburg, 1997)

Stem rust infection, %	The weight of 1000 grains		
	grams	% to a control	losses, %
0–10	36.3	100	–
20–40	30.7	84.5	15.5
50–60	26.3	72.4	27.6
70–100	23.3	64.2	35.8
Sd, g	**0.16**	–	–
LSD05, g	**0.8**	–	–

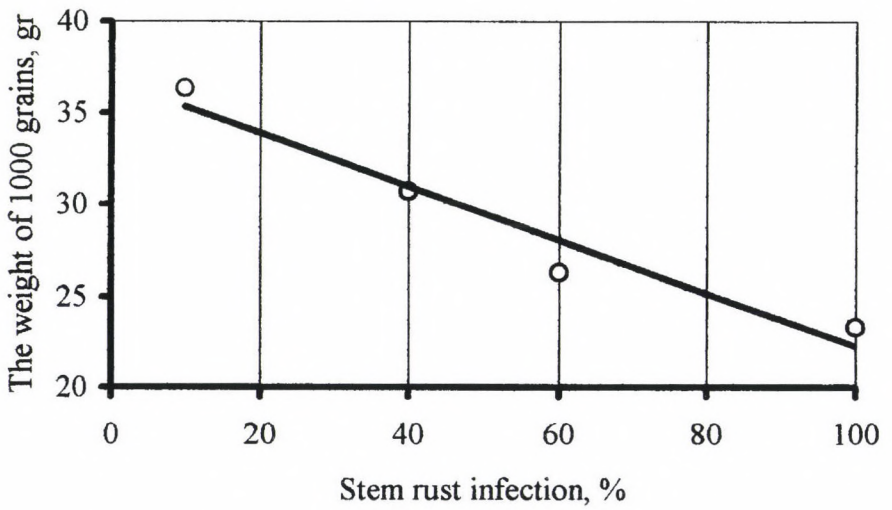


Fig. 1. The weight of 1000 grains for short-stem plants Getera 3 differing in degree of stem rust infection

The diagram shows the increase in degree of stem rust infection on susceptible plants for each 10% result in weight of 1000 grains loss for 1.5 grams.

There were cases of high tolerance in certain plants, for which loss in 1000 grains weight was no more than 20% under 70–100% level of infection. The cases of low tolerance were noted, too, when 1000 grains weight loss was up to 80% under the same degree of infection. It is necessary to note, that at combined infection of plants with stem rust (70–100%) and stem rot caused by *Fusarium culmorum* (W. G. Sm.), 1000 grains weight loss was more than 55% in comparison with control. Such plants have not been analysed in our research of stem rust effects to yield.

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Antagonistic Activity of Fungal Flora Isolated from Pepper Phylloplane on Post-Harvest Pathogens of Pepper *(Capsicum annuum)*

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Of the 29 different phylloplane microflora isolated from leaves, flowers and fruits of actively growing pepper *(Capsicum annuum)*, only three species of *Trichoderma* and *Rhizopus* strains were found to have antagonistic activities to *Aspergillus niger, Rhizopus nigricans, Penicillum citrium* and *Oidium* sp., the post-harvest pathogens isolated from rotted pepper fruit. The *in vitro* screening showed that the *Trichoderma* species had moderate to strong antagonistic activities to the post-harvest pathogens by inhibiting their growth in culture. The result showed that the mode of antagonism was by nutrient competition, though observation in some cases showed mycoparasitism. Successful antagonism occurred when the antagonists were inoculated several hours before the pathogens.

Keywords: antagonistic, fungal flora, post-harvest, pathogen, pepper.

Plant surface is a natural habitat representing a heterogeneous population of microbes. They are brought on the plant surface by air and there is a continuous interaction among the microbial metabolism, host metabolism, physical factors and the ecological niche of the leaf (Sharma and Mukereji, 1974a). Due to this established interaction, plants in a natural state possess a relatively stable biological balance of the microbes on their surface.

These microbes which are subject to various environmental fluctuations and stresses develop in response to factors such as pre- harvest and post-harvest activities over a growing period and may be pathogenic. Some of these organisms may prevent pre-harvest and post-harvest pathogenic infection of plants (Baker and Cook, 1974). There are many reports on the activities of antagonistic microorganisms on control of pathogenic fungi and bacteria of some crops such as apple, citrus, tomato (Janisiewicz, 1988; Roberts, 1990; McLaughlin et al., 1992; Latunde-Dada, 1993; Turhan and Grossman, 1994).

It is against this back ground that the present study was undertaken, to examine the activity of fungal micro-flora occurring naturally on pepper plant. Specifically assess the potential for the biological control of the post-harvest rot pathogens of pepper, an important economic condiment and spice in Nigeria. Pepper is known to have a high storage/post-harvest losses of up to 25% (Atanda et al., 1990) and the only keeping quality or preservation for long storage is by drying in the sun.

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Materials and Methods

Pepper *(Capsicum annuum)* seed were sown in a tray and transferred after four weeks to the field and were planted in four blocks at the experimental plots of Dept of Agronomy, University of Ibadan. The entire seedling was watered daily. At flowering stage until maturity/fruiting, leaves, flowers and fruits were collected randomly at two weeks intervals for two seasons in 1994 and 1995. Isolation of the epiphytic fungi were similar to those of (Janisiewicz, 1988; Roberts, 1990; McLaughlin et al., 1992). Each set of the plant parts was soaked separately in sterile distilled water. The beakers were then shaken on mechanical shaker for 30 minutes. One ml each of the washing suspension was plated on various selective media (malt-extract agar, potato dextrose agar, yeast extract agar, Czapek agar, and corn meal agar) in a 9-cm Petri dish. Incubation was done at room temperature for the required duration and this ranges from 1 to 7 days, to allow the growth of all organisms.

Each micro-organism was sub-cultured into separate sterile agar plates to obtain pure cultures of the isolate from the mixed cultures. Stock cultures of all the pure isolates were then made and kept in McCartney bottles and used during subsequent experiments. The isolates were tested in culture for antagonistic activity against the isolated post-harvest pathogens.

Isolation of the rot pathogens

Potato dextrose agar and corn meal agar were used to isolate the pathogens. The rot affected samples of pepper fruits were surface sterilized with 95% ethanol cut into 1 mm cubes, washed with several changes of sterile distilled water and blotted dry with filter paper. The cut pieces were then plated on agar in 9-cm Petri dish. Incubation was done at room temperature for periods ranging from 1 to 7 days to allow for the growth of different pathogens. Each pathogen was then subcultured into separate sterile agar plate to obtain pure culture. Stock cultures of the pathogens were then prepared and placed in McCartney bottles. The identifications of the fungi were done at International institute for tropical Agriculture, Ibadan, Nigeria, with reference to the works' of Barnett and Hunter (1987) and Raper and Fennel (1965).

Screening for antagonistic activity in vitro

Each organism isolated was paired with each of the pathogen to evaluate their antagonistic potentials. Pairing was done on potato dextrose agar (PDA) contained in 9-cm Petri dish as follows: (i) five-millimeter plug of the antagonist was placed at the edge of the 9-cm Petri dish and after 24 h the pathogen was placed at the opposite edge of the same Petri dish. (ii) The pathogen was placed at the edge of the petri dish 24 h before the antagonist, (iii) the antagonist and the pathogen were inoculated at the opposite edge of the plate simultaneously. There was control for each of these treatments with each treatment having three replicates. The dixenic cultures were incubated at 28 + 2 °C for 14 days and observation on colony growth of both antagonist and pathogen were done every 3 days

taking average measurements. Evaluation of antagonistic activity, the width of the inhibition zone and mycoparasitic development were done using the modified scale according to Turhan and Grossman (1994).

Treatments were laid out as a complete randomized design with three replicates.

Results

Twenty-nine fungal isolates were obtained from pepper fruits, leaves and flowers *(Table 1)*. Some of these epiphytes were common moulds, while others were field to harvest/storage saprophytes or pathogen such as *Fusarium* sp., *Botryodiplodia theobromae* and *Macrophomina* sp. The isolated pathogens from the rotted fruits were *Rhizopus nigricans, Aspergillus niger, Penicilium citrium* and *Oidium* sp. Of the isolated epiphytic

Table 1

Micro-flora isolated from phylloplane of actively growing pepper plants

Plant Parts		
Leaves	Flowers	Fruits
Botryodiplodia theobromae	*Aspergillus niger*	*Mucor* sp.
Macrophomina sp.	*B. theobromae*	*Rhizopus* sp.
Fusarium solani	*Unidentified*	*B. theobromae*
Trichoderma sp.	*Macrophomina* sp.	*Penicillium* sp.
Aspergillus aculentus	*Trichoderma* sp.	*T. pililuferum*
T. pililuferum	*Mucor spinosus*	*T. harzianum*
Fusarium solani	*A. flavus*	*Fusarium* sp.
B. theobromae	*F. solani*	*T. longibracitum*
Unidentified sp.	*Rhizopus oryzae*	
Penicillium sp.	*Aspergillus* sp.	
Macrophomina sp.		

fungal flora only four isolates inhibited the growth of the isolated post-harvest pathogens *(Table 2)*. These were identified as a strain of *Rhizopus* and three *Trichoderma* sp., i.e. *T. harzianum, T. piluliferum* and *T. longibrachiatum* whose, mode of antagonism were by nutrient competition. *T. harzianum* showed moderate to good inhibition to all the rot pathogens. They were covered between 4.1 to 6.60 cm *(Table 2)* of the plates when inoculated between 24 to 36 hours before the pathogen and weak inhibition when inoculated simultaneously with the pathogen. The only exception was the case of *A. niger* where *T. harzianum* seem to parasitise the pathogen, and strong inhibition when both before and with simultaneous inoculation. *T. piluliferum* was more active only when inoculated long before the pathogens and showed a very strong inhibition to all the pathogens *(Table 2)*. Though it produced green metabolites, when in culture with the

pathogens, this metabolite do not seem to be inhibitory to the pathogens. *T. longibrachiatum* also showed very strong inhibition when inoculated long before the pathogens and weak inhibition when inoculated simultaneously *(Table 2)*. The *Rhizopus* strain showed a moderate inhibition to all the rot pathogens except that of *R. nigricans* when inoculated long before the pathogens, but very weak inhibition when inoculated simultaneously *(Table 2)*. The statistical analysis showed that there is no significant difference in the effect of the antagonist against the pathogens at 5% probability level. There is, however, a significant difference among the 3 different ways of pairing as regards their effect in successful antagonism.

Table 2

Antagonistic activity of naturally occurring fungal flora isolated from the pepper phylloplane against rot pathogens

Isolated fungi	Pathogens isolated from rotted pepper			
	Rhizopus nigricans	*Aspergillus niger*	*Penicillium citrium*	*Oidium* sp.
Trichoderma harzianum	5	5	5	7
Trichoderma pililuferum	4	5	6	6
Trichoderma longibrachiatum	5	6	4	6
Rhizopus sp.	3	7	5	7

Key:
0 = No inhibition visible, pathogen grew round the whole Petri dish.
1 = The antagonist covered between 1.5–3.0 cm of the plate.
2 = „ „ „ 3.1–4.00 cm of the plate (weak inhibition)
3 = „ „ „ 4.1–5.0 cm of the plate (weak inhibition/antagonism)
4 = „ „ „ 5.0–6.50 cm of the plate (moderate inhibition/antagonism)
5 = The antagonist covered between 6.60–7.90 cm of the plate (good inhibition/antagonism)
6 = The antagonist covered between 8.0–8.90 cm of the plate (strong inhibition/antagonism)
7 = The antagonist covered between the whole plate (very strong inhibition/ antagonism)

Discussion

The *Trichoderma* species and the *Rhizopus* strain of the 29 different fungal floral isolated from the phylloplane of the pepper plant appeared to be antagonistic against the four isolated post-harvest fruit rot pathogens of pepper. All the organisms grew at ambient room temperature of 28 + 2 °C. The four isolate were inhibitory to the rot pathogens *in vitro*. *In vitro* tests can be used to screen many organisms to get information on their inhibitory activity on pathogens (Schiewe and Mendgen, 1992). Of the three *Trichoderma* spp. isolated, *T. harzianum* is a well-know antagonist (Elad et al., 1980). The other *Trichoderma* spp. that have been used successfully as bio-control agents include *T. hamatum, T. viride* and *T. konigii* (Latunde-Dada, 1993). In this study the three-*Tricho-*

derma spp. exhibited nutrient competition as their mode of action in the *in vitro* control of the rot pathogens. When the antagonist were inoculated several hours before the pathogens, the antagonism was strong due to faster growth of the antagonists in colonizing more space on the plate than the pathogens, but the antagonistic activities were moderate or weak when they were inoculated simultaneously, or pathogens before antagonists.

The mode of action of *T. piluliferum* could be by antibiosis because the antagonist produce a green metabolite that covered all part of the agar but there was no visible evidence of antibiosis. The mechanisms of the antagonist against the pathogens observed in this study was similar to the result obtained by (McLaughlin et al., 1992; Biles et al., 1990). They noted that some antagonist may affect a pathogen by more than one mode of action and invariably broaden their capability for bio-control. Our results showed that the isolated antagonistic micro-flora were effective in controlling the growth of the pathogen in culture by nutrient competition. Micro-organism can inhibit the development of a pathogen in culture by nutrient competition, without showing effects on the growth of the pathogen in culture and are not detectable by *in vitro* screening methods (Roberts, 1990; McLaughlin et al., 1992).

The statistical analysis showed that any of the three species of *Trichoderma* is a good antagonist. The inoculation of the antagonist before the pathogens was better than after or concurrently. This agreed with the work of Campbell (1988) who believed that there are no bio-control agents that have enough competitive ability to displace an already established pathogen. The time lapse between inoculation of the antagonist and the pathogen contributed to the success recorded with the antagonist against the pathogen. This is similar to the observation of (Janisiewicz, 1988; Roberts, 1990) on the importance of time-lapse between the arrival of the antagonist and later pathogen on the phylloplane. According to them this allows adequate increase in cell concentration and subsequent colonization by antagonist before the arrival of the pathogen. The significance of this study is that *T. piluliferum* and *T. longibrachiatum* that were found to be antagonistic to the rot pathogens according to the authors knowledge have not been reported in literature to be antagonistic to any pathogen.

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The Influence of Durable Subcultivation of Leaf Rust Pathogen on Compatibility with Wheat Varieties

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Natural population of leaf rust was subcultured 10 times on seedlings of 5 spring varieties – 'Biserka', 'Inia 66', 'Saratovskaja 29', 'Djemchujina Zavoljia' (all have some level of partial resistance in seedling and adult stages) and 'Thatcher' (susceptible control). Final subpopulations were twice reproduced on susceptible variety and then used to inoculate all experimental genotypes. The interaction of genotype with subpopulation was highly significant as well as correlation between final resistance of variety and nonspecific aggressiveness of its subpopulation. All varieties except 'Inia 66' lost their resistance but only for 'Biserka' this loss was specific and followed by increasing of corresponding subpopulation aggressiveness. The difference between cropping history and obtained results are probably explained by different genetic systems determined seedling and adult partial resistance to leaf rust and/or different expression of the same genes at different stages of plant growth.

Keywords: wheat, leaf rust, partial resistance.

The constant problem of plant breeding for phytopathogen resistance is a regulary loss of resistance of commercial varieties due to multiplication of causal agent's races adapted to them.

The durability of resistant of a variety depends on its genotypes. There exist varieties with stable resistance during decades despite significant selection pressure for parasite races virulent to it, while other varieties rapidly lose resistance under the same epidemiological conditions.

It is well known that durable resistance cannot be distinguished easily from quickly lost neither according to phenotype expression nor genetic control (Johnson, 1985; Parlevliet, 1978; Odintsova and Mikhailova, 1988). The single reliable criterion of resistance durability is variety cropping history. The search of another criteria is obviously necessary for plant breeding.

One of the perspective approaches may be durable cultivation of pathogen on partially resistant genotype and comparison of initial and final levels of resistance.

This work is one of attempts to use this approach for studying the possible durability of wheat resistance to leaf rust.

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Materials and Methods

In the experiments we used two varieties of soft wheat (*Triticum aestivum* L.) that have a reputation of having durable partial resistance to wheat leaf rust (*Puccinia recondita* Rob. ex Desm. f. sp. *tritici*) 'Biserka' (Donchev, 1984) and 'Saratovskaja 29'. 'Saratovskaja 29' was grown in Russia on large areas during long time and nevertheless kept its slightly expressed resistance. Besides we used moderately resistant varieties 'Inia 66' and 'Djemtchugina Zavolja', previously identified in our work. 'Thatcher' was sown as susceptible control.

Firstly the varieties were evaluated in the first leaf stage to be sure that their resistance was expressed, at least partially, in seedling phase. Then on each of the five varieties natural leaf rust population was subcultured, and after 10th subculturing we obtained 5 subpopulations, corresponding to 5 used wheat genotypes. Each subpopulation was twice reproduced on susceptible variety to eliminate effect of physiological adaptation and then used for resistance evaluation of experimental varieties.

As a criterion of parasite adaptation abilities we used comparision of the compatibility with "own" subpopulation and that with other ones.

Subcultivations of pathogen were made on leaf segments placed on benzimidazol solution (60 ppm) (Mikhailova and Tyryshkin, 1988) according to method worked out for *Bipolaris sorokiniana* (Tyryshkin and Voronkova, 1997).

The degree of compatibility of subpopulations with experimental samples was determined on leaf segments as number of pustules developed on leaf segments after inoculation with spore suspension. Before inoculation of each spore concentration of subpopulation was counted in hemocytometer and diluted to 10^4 uredospores/ml. Pustules were counted on 20 leaf segments in two or three independent experiments and data were calculated according to ANOVA test.

To eliminate the possible effects of some deviation from equal spore concentration, indexes of pustule numbers as a % of that on 'Thatcher' were used. The number of pustules of subpopulations on all tested varieties was evaluated as an index of nonspecific aggressiveness.

Results

All experimental wheat genotypes possess moderate or weak ('Saratovskaja" 29) expressed resistance comparing with 'Thatcher' and this resistance in all varieties, except 'Saratovskaja 29', is expressed considerably more in the flag-leaf than in the seedling stage *(Table 1)*. The differences in results of experiments *I* and *II* are not principal and can be explained by differences in parasite populations used and/or experimental conditions.

The evaluation of degree of compatibility after 10 cycles of reinoculation on resistant varieties showed that variety – subpopulation interaction was significant ($P < 0.01$) *(Table 2)*. The number of pustules of "own" subpopulation was highly and significantly correlated with nonspecific aggressiveness of this subpopulation ($r = 0.99$; $P < 0.01$).

Table 1

The number of pustules of leaf rust on leaf segment of 5 experimental wheat varieties after inoculation with natural population of *Puccinia recondita*

Variety	Experiment I, 1994				Experiment II, 1998	
	Seedling stage		Flag-leaf stage		Seedling stage	
	Number	% Tc*	Number	% Tc	Number	% Tc
Biserka	187	52	13	12	140	46
Saratovskaja 29	206	58	48	44	206	69
Djemchujina Zavoljia	198	55	18	16	210	70
Inia 66	191	53	6	5	199	66
Thatcher	358	100	110	100	302	100

*number of pustules on the variety in % of that on 'Thatcher'

Table 2

Result of ANOVA test for interaction of 5 wheat varieties with 5 leaf rust subpopulation

Variation	Degrees of freedom	Sums of squares	Mean squares	Ffact.	Ftabl.	
					5%	1%
In pathogen population	4	7565	1891	84.8	2.46	3.51
In plant genotypes	4	1480	370	16.59	2.46	3.51
In interaction of pathogen and plant genotypes	16	1098	68.6	3.08	1.85	2.36
Residual	475	10573	22.3	–	–	–
General	499	20716	–	–	–	–

This phenomenon was earlier noted by Ellingboe (1975) but in our work we used two cycles of populations reproduction on susceptible variety before compatibility assessment to eliminate the possible physiological adaptation. Nevertheless, we cannot exclude that the cause of coincidence of subpopulations aggressiveness with resistance expression of corresponding varieties is residual physiological pathogen adaptation. Genetic adaptation is possible, too, if reproduction on resistant varieties leads to elimination of the most aggressive fungal isolates.

Crossed inoculation of all varieties with all subpopulations revealed that number of pustules on leaf surface unit expressed in % of that on 'Thatcher' on varieties 'Inia 66', 'Saratovskaja 29' did not change principally comparing with their initial level *(Table 3).* But for 'Biserka' and 'Djemtchugina Zavolja' we found increasing compatibility with "own" subpopulation as compare with their initial level. This result for 'Djemtchugina Zavolja' is in contradiction with low aggressiveness of fungal subpopulation reproduced on this variety. Taking into account the close correlation of variety resistance with non-

Table 3

Varieties resistance to "own" subpopulation of leaf rust and average aggressiveness of subpopulations

10th subpopulation from variety	Number of pustules on				
	'own' variety	Thatcher	%	all varieties**	%
Biserka	388	109	1436	135	
Saratovskaja 29	139	158	88	635	109
Djemchujina Zavoljia	88*	65	135	407	108
Inia 66	89*	128	70	595	75
Thatcher	277	100	1175	107	

* significant difference from 'Thatcher' ($P<0.05$)
** index of nonspecific aggressiveness

specific aggressiveness of its subpopulation, we have to doubt in the number of pustules of 'own' subpopulation on 'Djemtchugina Zavolja' presented as % of that on 'Thatcher'. We consider that only 'Biserka' undoubtedly can be regarded as having lost its resistance because this is supported by two indexes: number of pustules on it as compare with 'Thatcher' and nonspecific aggressiveness of subpopulation, reproduced on it.

The only variety that kept its high level of resistance comparing with 'Thatcher' was 'Inia 66' and its subpopulation was the least aggressive *(Table 3)*.

For our purposes it was necessary to divide specific fungus adaptation to resistant genotypes and influence of variety resistance on nonspecific subpopulation aggressiveness. For this we used number of pustules on leaf surface unite divided to average number of pustules of this subpopulation on all varieties. In all cases, except 'Biserka' and 'Inia 66' these indexes were close to 1.0. Consequently, specific adaptation of population during durable reinoculation we observed only for variety 'Biserka'.

Discussion

We observed clear differentiation for parasite population ability to adapt to resistant varieties: adaptation to 'Biserka' was expressed dramatically and for other varieties was absent or questionable.

Moderately leaf rust resistant variety 'Biserka' Donchev (1984) defined as durable resistant on the basis of its pedigree as well as durable experience of commercial use on Balkan of this variety and its relatives. Differences of our data and this affirmation can be explained most likely by the fact that we studied the seedling component of its resistance. This component is a minor part of overall resistance expressed in field at flag-leaf stage *(Table 1)*.

We do not have any explanation of genetic nature of decreasing nonspecific aggressiveness of population on partially resistant 'Inia 66', the single variety that undoubtedly kept its resistance, but the same result we have received on *Bipolaris sorokiniana* – causal agent of dark brown leaf spot blotch of wheat (Tyryshkin and Voronkova, 1997).

We suppose that durable reinoculation of phytopathogene populations may be useful for prediction of durable variety resistance and for evaluation of sources with possibly durable resistance. It should be noted that the method allows only to eliminate the genotype in case of experimentally observed adaptation. If it is absent, it does not mean that it will not take place in nature in "agronomic future". But we suppose it to be useful, because breeding for durable resistance is time-consuming, and wrong selection of donors is very undesirable.

Our data on increasing of nonspecific aggressiveness after population reproduction on susceptible varieties (see also Tyryshkin and Voronkova, 1997) show that their commercial use is harmful for agriculture not only because of their yields losses after diseases development but also because moderately resistant varieties are attacked severely as a result of nonspecific pathogene aggressiveness increase.

Our data also suggest, that discussed approach about breeding of multilane varieties ("dirty crop approach") can be unsuccessful if as resistant component will be used partially resistant genotypes.

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Influence of Organic Amendments on the Antagonistic Population and Their Effect on Sclerotial Wilt of Jasmine

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Sclerotial wilt of jasmine incited by *Sclerotium rolfsii* is a serious menace causing complete death of seedlings and grownup plants. The organic amendments were evaluated for their efficacy in managing the sclerotial wilt disease and on the antagonistic population. The results revealed that neem cake significantly inhibited the growth of *S. rolfsii* under *in vitro* conditions. The lowest sclerotial number, sclerotial dry weight and sclerotial germination were recorded in neem cake amended culture medium. Amending the soil with neem cake also effectively controlled the sclerotial wilt disease under greenhouse conditions. The increase in the population of beneficial fungi, bacteria and actinomycetes in the amended soils was found to be significantly more than the population in the non-amended soils. Neem cake-amended soil recorded the maximum *Trichoderma* spp. and *Pseudomonas fluorescens* population. The inoculum levels of *S. rolfsii* were found to be lower in neem cake-amended soil.

Keywords: jasmine, *Sclerotium rolfsii*, organic amendments, antagonistic population.

Jasmine (*Jasminum sambac* L.) occupies a major share in the flower markets which contribute much to the national economy in India. It plays a major role in the concrete oil extraction industry. *Sclerotium rolfsii* Sacc. causes extensive damage in various plant species in warm regions of the world (Punja, 1985). However, this is the first report of *S. rolfsii* infecting jasmine in India. Sclerotial wilt is a serious soil-borne disease in jasmine. White mycelial strands appear around infected regions of the leaf, stem and root which gradually become pale and as a result the plants get wilted. In later stages sclerotial bodies are developed and the stem gets girdled resulting in toppling of infected seedlings at the collar region and death of the plants occurred. The seedlings as well as the pathogen establish well in the silty loam soil (Alice et al., 1998). Control efforts of this dreaded pathogen have met with limited success, due to its extensive host range, prolific growth and ability of the pathogen to produce large number of sclerotia that may persist in soil for several years and posses high resistance to microbial attacks (Shahi et al., 1998). The soil is a complex store house of the different microorganisms, and the chemical control measures involving the use of broad spectrum fungicides bring about imbalance in the microbial community. Thus, the use of chemicals for the control of diseases caused by sclerotial pathogen under field conditions is not much encouraging. The growing realiza-

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tion that plant and their products have the potential in providing materials that are deadly to fungi has prompted a renewed look at these natural biocides. In response to environmental and health concerns due to extensive use of pesticides, there is a considerable interest in finding alternative control approach involving the use of biocides in integrated pest management strategies for crop diseases.

Organic amendments are recommended as biological means to reduce the incidence of several soil-borne diseases (Singh et al., 1979; 1980; 1991; Sekhar, 1985; Alice et al., 1998; Raguchander et al., 1998; Scholte and Lootsma, 1998; Meena and Muthusamy, 1999). It is a cheap, practicable and effective method of disease control. The decomposition of organic matter helps in alteration of physical, chemical and biotic conditions of the soil. The altered conditions reduce the inoculum potential of the pathogens. In addition, it improves soil structure which promotes root growth of the plant. Various biochemicals released during decomposition induce disease resistance/tolerance on the root surface as well as in the tissues when absorbed (Hundekar et al., 1998). Ability of several organic amendments to promote activity of indigenous antagonists resulting into plant diseases control cannot be denied. In the present study, nine organic amendments are evaluated for their efficacy against *S. rolfsii* in jasmine.

Materials and Methods

Isolation of the pathogen

Various isolates of *S. rolfsii* were isolated from stem portions of infected jasmine plant using potato dextrose agar (PDA) medium. The isolates were mass-multiplied in sand-maize medium (19:1 sand : maize) (Riker and Riker, 1936). The pathogenicity of the isolates was tested by artificially inoculating the fungus mass-multiplied in sand maize medium at 5% level in pot soil (sand : red earth : FYM @ 1:1:1w/w/w) and planted with jasmine seedlings. The virulent isolate was selected based on per cent disease incidence (data not shown), re-isolated using PDA medium and used for further studies.

In vitro *screening of organic amendments against* S. rolfsii

Different organic amendments viz., Farm Yard Manure (FYM), compost, *Tephrosia purpurea* (kolingi), neem cake, coconut cake, pungam cake, castor cake and groundnut cake were dried at room temperature (27±2 °C) for three days, broken into small pieces and powdered thoroughly with pestle and mortar, sieved through a screen (2 mm porosity) and homogenated in sterilized distilled water at the rate of 1 ml/g. After 24 h, extract was obtained by squeezing the homogenate through fourfolds of muslin cloth. The extract was centrifuged and the supernatant was decanted into a 250 ml flask and filtered through Seitz bacteriological filter. This formed the standard extract solution (100%). The fungitoxicity of the extracts of various organic amendments was tested by poisoned food technique. The extract was further diluted to 10% concentration by adding requisite amount of PDA

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medium. The extract was mixed with PDA medium and twenty ml medium was poured into each of the sterile Petri dishes. The incorporation of carbendazim (0.1%) in the medium was used for comparison. After solidification, a 9 mm culture disc of *S. rolfsii* (5 day old) was inoculated at the centre of the plate. The PDA medium without incorporating the extracts and inoculated with pathogen served as the control. The plates were incubated at 27±2 °C for five days and the radial growth of the pathogen was measured. The number of sclerotia formed was counted after ten days of incubation. To assess the sclerotial germination, 10 treated sclerotia were placed on PDA medium at equidistance and the germination percentage was recorded after 48 h. Three replications were maintained for each treatment.

Effect of organic amendments against sclerotial wilt of jasmine

A pot culture experiment was conducted to assess the efficacy of organic amendments against sclerotial wilt of jasmine. Earthen pots of size 30 x 30 cm were filled with six kg of sterilized sandy loam soil. The organic amendments viz., FYM (12.5 t/ha), decomposed coir pith (12.5 t/ha), compost (12.5 t/ha), coconut cake (150 kg/ha), neem cake (150 kg/ha), pungam cake (150 kg/ha), castor cake (150 kg/ha), groundnut cake (150 kg/ha) and *Tephrosia purpurea* (Kolingi) (10 t/ha) were applied basally to the pot-soil 15 days prior to the transplanting of seedlings and allowed to decompose. The pathogen mass-multiplied as described earlier in sand-maize medium was inoculated to the pots at 5% level (w/w). Jasmine seedlings were then transplanted. Control was maintained by filling the pots with non-amended soil. Soil-drenching with carbendazim (0.1%) was kept as check. Five pots constitute one replication and three replications for each treatment were maintained. Number of plants infected per treatment was recorded at 60th day after planting. Soil sample was collected from each treatment separately for estimation of microbial population and enumeration was done at three different periods viz., 20, 40 and 60 days after the application of amendments.

Effect of organic amendments on the antagonistic population

Microbial population in the amended and non-amended soil was estimated by following serial dilution method. Dilutions used for microbial enumeration include 10^{-5} for bacteria, 10^{-4} for fungi and 10^{-3} for actinomycetes. Media used for microbial enumeration were Nutrient agar medium (Rangaswami, 1972) for total bacteria, Rose Bengal agar medium (Martin, 1950) for total fungi and Kusters agar medium (Kuster and Williams, 1964) for actinomycetes. Microbial count was made on the 2nd day for bacteria, 7th day for fungi and on the 10th day for actinomycetes.

The enumeration of *Trichoderma* spp. population and *Pseudomonas fluorescens* population was done by using *Trichoderma* Special Medium (TSM) (Elad and Chet, 1983) and King's B medium (King et al., 1954), respectively. The dilutions used were 10^{-4} and 10^{-5}, respectively. Microbial count was made on the 5th day and 2nd day for *Trichoderma* and *P. fluorescens,* respectively.

Effect of organic amendments on the population of S. rolfsii

To assess the population of *S. rolfsii* in the soil, about one g of soil sample was collected in each pot at different periods after incorporation of amendments and the number of sclerotia per g of soil was counted.

Results

In vitro screening of organic amendments against *S. rolfsii* revealed that neem cake and carbendazim (0.1%) significantly inhibited the mycelial growth of the pathogen by recording 3.6 cm and 3.4 cm radial growth, respectively, while there was 8.8 cm growth of mycelium in the control. This was followed by FYM, compost and castor cake which showed the mycelial growth of 3.8, 4.4 and 5.2 cm, respectively. The mycelial growth was the maximum (6.6 cm) with 10% leaf extract of *T. purpurea (Table 1).* The lowest sclerotial number of *S. rolfsii* was produced by carbendazim (0.1%) (22.8) which was found to be on par with neem cake (23.4) and FYM (28.6) as against 86.8 in the control. The sclerotial number was found to be the maximum in *T. purpurea* (58.6) *(Table 1).*

Table 1

In vitro evaluation of organic amendments against *S. rolfsii*

Extracts (10%)	Mycelial growth of *S. rolfsii* (cm)	Sclerotial number	Sclerotial dry weight (g)	Sclerotial germination (%)
Farm Yard Manure (FYM)	3.8	28.6	0.9	20.0
Compost	4.4	42.4	1.3	40.0
Tephrosia purpurea (kolingi)	6.6	58.6	2.1	53.3
Neem cake	3.6	23.4	0.8	20.0
Coconut cake	5.8	49.6	1.6	43.3
Pungam cake	6.2	54.2	1.8	50.0
Castor cake	5.2	46.5	1.5	36.7
Groundnut cake	6.4	52.4	2.0	53.0
Carbendazim (0.1%)	3.4	22.8	0.8	20.0
Control	8.8	86.8	2.8	100.0
CD (p = 0.05)	1.5	9.0	0.5	10.2

The data are mean of three replications

Neem cake and carbendazim (0.1%) recorded the lowest sclerotial dry weight of 0.8 g which were on par with FYM (0.9 g). Sclerotial dry weight of 2.8 g was recorded in the control and *T. purpurea* leaf extract (10%) exhibited 2.1 g *(Table 1).* Neem cake, FYM and carbendazim (0.1%) were equally effective in reducing the sclerotial germination of *S. rolfsii* by exerting 20% sclerotial germination. Castor cake and compost ranked next which recorded 36.7% and 40.0% sclerotial germination as against 100% in the control *(Table 1).*

Table 2

Effect of organic amendments against sclerotial wilt of jasmine

Amendments	Disease incidence (%)
Farm Yard Manure (FYM) (12.5 t/ha)	33.3
Decomposed coir pith (12.5 t/ha)	40.0
Compost (12.5 t/ha)	33.3
Tephrosia purpurea (kolingi) (10 t/ha)	66.7
Neem cake (150 kg/ha)	26.7
Coconut cake (150 kg/ha)	46.7
Pungam cake (150 kg/ha)	60.0
Castor cake (150 kg/ha)	46.7
Groundnut cake (150 kg/ha)	66.0
Carbendazim (0.1%)	23.3
Control – Pathogen inoculated	80.0
Control – Pathogen uninoculated	20.0
CD (p = 0.05)	12.3

The data are mean of three replications

Table 3

Effect of organic amendments on the total fungal and *Trichoderma* spp. population

Treatments	Total fungal population (cfu x 10^4/g soil)			*Trichoderma* spp. population (cfu x 10^4/g soil)		
	D1	D2	D3	D1	D2	D3
Farm Yard Manure (FYM) (12.5 t/ha)	20.0	23.3	25.0	5.3	6.3	7.0
Decomposed coir pith (12.5 t/ha)	18.7	20.0	24.0	5.0	6.0	6.0
Compost (12.5 t/ha)	18.0	21.3	24.0	5.0	6.0	6.0
Tephrosia purpurea (kolingi) (10 t/ha)	12.0	15.7	18.0	3.0	4.3	5.3
Neem cake (150 kg/ha)	20.3	26.7	29.0	5.3	6.0	8.3
Coconut cake (150 kg/ha)	15.0	19.0	21.0	4.7	5.0	5.7
Pungam cake (150 kg/ha)	15.3	19.0	21.0	4.0	5.0	6.0
Castor cake (150 kg/ha)	16.0	19.0	22.0	4.0	4.7	6.0
Groundnut cake (150 kg/ha)	16.0	19.3	22.7	4.7	5.3	6.3
Carbendazim (0.1%)	8.3	11.0	13.0	3.0	4.0	4.3
Control – Pathogen inoculated	8.3	10.0	12.7	3.0	4.0	4.3
Control – Pathogen uninoculated	8.3	11.0	13.7	3.0	4.0	5.0
CD (p = 0.05)						
Treatments = 3.2						
Days = 1.8						
Treatments x Days = 5.4						
CD (p = 0.05)						
Teatments = 0.6						
Days = 0.3						
Treatments x Days = 1.1						

D1 – 20 days after the application of organic amendments
D2 – 40 days after the application of organic amendments
D3 – 60 days after the application of organic amendments

In the present investigation, the infection of jasmine plants by sclerotial wilt pathogen was reduced in the amended soils as compared to the non-amended soils. The minimum average wilt infection of 23.3% was recorded with carbendazim (0.1%) and it was at par with neem cake-amended soils (26.7%). The other amendments viz., FYM, compost, decomposed coir pith, coconut cake, castor cake and pungam cake recorded 33.3, 33.3, 40.0, 46.7, 46.7 and 60% infection, respectively, whereas 80% infection was recorded with non-amended soils *(Table 2)*. Kolingi was the least effective in reducing the disease (66.7% infection).

The increase in the population of beneficial fungi, bacteria and actinomycetes in the amended soils was found to be significantly more than the population in the non-amended soils *(Tables 3, 4 and 5)*. The total fungal population gradually increased during the decomposition of organic amendments. It was the highest in neem cake-amended soils (29.0 x 10^4/g) followed by FYM-amended soils (25.0 x 10^4/g) at 60 days after the

Table 4

Effect of organic amendments on the total bacterial and *Pseudomonas fluorescens* population

Treatments	Total bacterial population (cfu x 10^5/g soil)			*P. fluorescens* population (cfu x 10^5/g soil)		
	D1	D2	D3	D1	D2	D3
Farm Yard Manure (FYM) (12.5 t/ha)	18.0	24.3	26.0	5.0	7.0	10.0
Decomposed coir pith (12.5 t/ha)	17.0	20.7	22.3	4.0	7.0	9.0
Compost (12.5 t/ha)	17.3	20.0	22.0	4.0	7.0	9.3
Tephrosia purpurea (kolingi) (10 t/ha)	12.3	14.7	16.0	3.0	5.0	7.0
Neem cake (150 kg/ha)	18.0	22.7	25.0	5.0	7.3	10.0
Coconut cake (150 kg/ha)	15.0	18.0	21.3	3.7	5.0	7.0
Pungam cake (150 kg/ha)	15.0	18.0	22.0	3.7	5.3	8.0
Castor cake (150 kg/ha)	16.3	18.0	19.0	4.0	6.0	8.0
Groundnut cake (150 kg/ha)	16.0	19.0	22.0	4.0	6.3	8.7
Carbendazim (0.1%)	9.0	12.0	14.0	3.0	4.0	4.3
Control – Pathogen inoculated	8.0	10.3	12.0	3.0	3.0	4.0
Control – Pathogen uninoculated	9.0	12.0	14.3	3.0	4.0	5.3

CD (p = 0.05)		CD(p = 0.05)	
Treatments	= 2.8	Teatments	= 0.6
Days	= 1.2	Days	= 0.2
Treatments x Days	= 4.6	Treatments x Days	= 0.9

D1 – 20 days after the application of organic amendments
D2 – 40 days after the application of organic amendments
D3 – 60 days after the application of organic amendments

application of organic amendments *(Table 3)*. *Trichoderma* population was the maximum in neem cake-amended soil (8.3 x 10^4/g) which was on par with FYM-amended soil (7 x 10^4/g) at 60 days after the incorporation of amendments *(Table 3)*. The total bacterial population gradually increased in neem cake and FYM-amended soil *(Table 4)*. *P.*

fluorescens population was the maximum both in neem cake and FYM-amended soil (10 x 10^5/g) followed by compost (9.3 x 10^5/g) and decomposed coir pith-amended soil (9 x 10^5/g) at 60 days after the incorporation *(Table 4)*. Carbendazim (0.1%) used for comparison did not have any effect on the antagonistic population.

The total actinomycetes population also gradually increased following the application of organic amendments. It was maximum in neem cake and FYM-amended soil (9 x 10^3/g) as against the lowest (3 x 10^3/g) in control at 60 days after the incorporation of amendments *(Table 5)*.

Table 5

Effect of organic amendments on the actinomycetes population

Treatments	Actinomycetes population (cfu x 10^3/g soil)		
	D1	D2	D3
Farm Yard Manure (FYM) (12.5 t/ha)	5.0	6.0	9.0
Decomposed coir pith (12.5 t/ha)	5.0	5.0	8.3
Compost (12.5 t/ha)	4.0	4.7	6.7
Tephrosia purpurea (kolingi) (10 t/ha)	3.0	3.7	5.0
Neem cake (150 kg/ha)	5.0	6.7	9.0
Coconut cake (150 kg/ha)	4.0	5.0	6.0
Pungam cake (150 kg/ha)	3.7	5.0	6.0
Castor cake (150 kg/ha)	4.0	4.7	6.0
Groundnut cake (150 kg/ha)	4.0	5.0	6.3
Carbendazim (0.1%)	2.0	3.0	3.3
Control – Pathogen inoculated	2.0	2.7	3.0
Control – Pathogen uninoculated	2.0	3.0	4.0

CD (p = 0.05)

Treatments = 0.3
Days = 0.2
Treatments x Days = 0.6

D1 – 20 days after the application of organic amendments
D2 – 40 days after the application of organic amendments
D3 – 60 days after the application of organic amendments

The inoculum levels of *S. rolfsii* were drastically reduced in the soil applied with organic amendments. The minimum sclerotial number (8.3) per g of soil was recorded in the neem cake-amended soil, followed by 12.7, 13.0, 13.0 and 16.0 per g of soil from FYM, compost, carbendazim (0.1%) and decomposed coir pith-amended soils, respectively, which were on par with each other and significantly more than that observed in non-amended soils (54.3) *(Table 6)*.

Table 6

Effect of organic amendments on the sclerotial number of *S. rolfsii*

Treatments	Number of sclerotia/g soil		
	D1	D2	D3
Farm Yard Manure (FYM) (12.5 t/ha)	22.0	18.3	12.7
Decomposed coir pith (12.5 t/ha)	24.3	20.0	16.0
Compost (12.5 t/ha)	24.0	20.3	13.0
Tephrosia purpurea (kolingi) (10 t/ha)	36.0	30.0	24.0
Neem cake (150 kg/ha)	22.7	16.0	8.3
Coconut cake (150 kg/ha)	30.0	24.7	20.0
Pungam cake (150 kg/ha)	32.3	26.0	22.3
Castor cake (150 kg/ha)	30.0	26.0	20.0
Groundnut cake (150 kg/ha)	34.0	29.3	23.0
Carbendazim (0.1%)	22.0	18.0	13.0
Control – Pathogen inoculated	40.3	48.7	54.3
Control – Pathogen uninoculated	–	–	–

CD (p = 0.05)

Treatments = 5.8
Days = 3.4
Treatments x Days = 10.6

D1 – 20 days after the application of organic amendments
D2 – 40 days after the application of organic amendments
D3 – 60 days after the application of organic amendments

Discussion

Several workers reported a reduction in the disease incidence in soil amended with various organic substrates (Dhingra and Sinclair, 1975; Satischandra et al., 1979; Sekhar, 1985). Organic amendments with oil cakes, extract of seeds and flowers of margosa have been found to decrease losses caused by different soil-borne plant pathogens (Singh and Singh, 1970; Goswami and Swarup, 1971; Worrigton and Corns, 1976).

In the present study, neem cake extract was found to be effective in inhibiting the mycelial growth and sclerotial dry weight of the pathogen under *in vitro* conditions. The inhibitory effect of the neem cake might be attributed to the presence of some antifungal ingredients (Gautam and Kolto, 1979). Similarly, Singh and Dwivedi (1990) reported that neem oil was the most effective organic amendment among the different plant oils and extracts tested against *S. rolfsii*. Shahi et al. (1998) found that margosa oil cake extract inhibited the mycelial growth of *S. rolfsii* under *in vitro* conditions. Gerard Ezhilan et al. (1994) found that pinnai oil cake extract prevented the sclerotial production and germination of *R. solani*. Thus, the inhibitory effect of concentrated oil cake extracts might be due to the presence of active principles.

It has been well documented that neem is endowed with biologically active antimicrobial compound(s) and many fungal pathogens have been found to be sensitive to these compound (s) (Tansey and Appleton, 1975; Singh et al., 1979; 1980; 1991). Earlier

we have found that neem cake was effective in reducing the rice sheath blight disease (Meena and Muthusamy, 1999). In the present study also, jasmine wilt incidence was greatly reduced in pot-soil amended with neem cake. Hundekar et al. (1998) reported that neem cake was found to be effective in reducing the inoculum levels of *M. phaseolina* and *F. moniliforme* thus resulted in minimum incidence of stalk rot of sorghum. Similarly, soil amended with neem cake significantly reduced the infection potential of *F. solani* in sunflower and combined application of neem cake with *Pseudomonas aeruginosa* or *Sterochospermum marginatum* resulted in greater biomass production when compared to separate application (Ehteshamul-Haque et al., 1998). Under field condition, a significant reduction in damping-off of tobacco incited by *Pythium aphanidermatum* was brought about by application of neem cake (Narayanaswamy et al., 1998). Padmodaya and Reddy (1999) reported that soil-application of FYM and neem cake was effective in reducing the seedling disease in tomato caused by *F. oxysporum* f. sp. *lycopersici.* Theradimani and Marimuthu (1993) found that soil-application of composted coirpith reduced pre- and post-emergence damping-off of chillies and dry root rot of blackgram. Combined application of organic amendments viz., FYM and neem cake with *T. viride* was effective in reducing the charcoal rot of soybean caused by *M. phaseolina* (Raguchander et al., 1998). In general, augment of soil with organic compost stimulates suicidal germination of fungal propagules; arrests the growth of germlings; causes lysis of hyphae; serves as substrate for antibiotic production and produces inhibitory volatile substances (Papavizas and Lumsden, 1980).

The present study also shows that the beneficial antagonistic microorganisms such as *T. viride* and *P. fluorescens* were higher in the soil amended with organic amendments viz., neem cake and farm yard manure. The increased microorganisms might have been implicated in the suppression of sclerotial wilt incidence in jasmine in addition to direct antifungal activity. Hundekar et al. (1998) and Linderman (1989) observed a net increase in population of bacteria, fungi and actinomycetes in soil incorporated with organic amendments. Hoitink (1980) reported that soil suppressiveness was due to enhanced native antagonists. Raguchander et al. (1998) reported that *T. viride* along with FYM and neem cake supported the production of maximum number of *T. viride* chlamydospores, better rhizobium nodules and reduction in sclerotial number of *M. phaseolina* and higher yield in blackgram. Scholte and Lootsma (1998) reported that farm yard manure alone or in combination with green manure crop (white mustard) reduced the *Rhizoctonia* stem canker of potato disease and increased the population of mycophagous soil organisms. Organic amendments act as a source of food and energy for soil beneficial biota which are very important for development of soil profile and maintenance of soil fertility. Multiplication of microorganism antagonistic to *S. rolfsii* in the organic amendment incorporated soil might have been responsible for the suppression of sclerotial bodies in the soil.

Hence, the organic amendments due to the presence of antifungal principles directly affect the soil-borne pathogens and indirectly augment the rhizosphere beneficial microorganisms which in turn inhibit the pathogenic organisms by various modes of biological control. To the best of our knowledge, this is the first report on the use of organic amendments for the management of sclerotial wilt of jasmine.

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Expression of a Rice Thaumatin-Like Protein (PR-5) Gene in a Baculovirus Expression System

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We previously isolated a full-length thaumatin-like protein (TLP) cDNA clone (D34) from a rice cDNA library prepared from RNA isolated from *R. solani*-infected rice plants. Furthermore, it has been demonstrated that transgenic rice plants that are constitutively expressing the TLP (D34) show enhanced resistance to *R. solani*. In order to purify and characterize this TLP, we expressed it (D34) in *Spodoptera frugiperda* (sf21) insect cells using a baculovirus expression system. Western blot analysis indicated that a 23-kDa protein cross-reacting with a bean TLP antibody was produced abundantly in the insect cells. However, this protein was not secreted into the culture medium. The N-terminal sequence of the protein (19 residues) showed high sequence similarity to thaumatin and TLPs of oat, barley, maize, grapes and tomato. The cleavage site for the TLP was determined as immediately upstream of the sequence ATFAITN.

Keywords: *Oryza sativa*, pathogenesis-related protein, N-terminal sequence.

Thaumatin-like proteins (TLPs) are a group of pathogenesis-related (PR) proteins (PR-5) that are induced in plants in response to infection by pathogens (Bryngelsson and Green, 1989; Velazhahan et al., 1998), osmotic stress (Singh et al., 1987; LaRosa et al., 1989), treatment with abscisic acid (Singh et al., 1989), treatment with ethylene (Koiwa et al., 1994), treatment with *Pseudomonas fluorescens* (Meena et al., 2000), application of salicylic acid (Jung et al., 1993) and wounding (Neale et al., 1990; Brederode et al., 1991). Since purified TLPs from plants exhibit antifungal activity against a variety of fungi (Roberts and Selitrennikoff, 1990; Vigers et al., 1991), TLPs are postulated to play a role in plant defense. It has been demonstrated that transgenic plants constitutively expressing high levels of PR-5 proteins showed an enhanced disease resistance (Liu et al., 1994; Chen et al., 1999; Datta et al., 1999). TLPs have been isolated or cloned from many monocot and dicotyledonous plants (Velazhahan et al., 1999). Velazhahan et al. (1998) isolated a full-length clone of TLP cDNA (D34) from a rice cDNA library prepared from RNA isolated from *R. solani*-infected rice plants. The cDNA contained an open reading frame of 693 bp encoding 231 amino acids with a predicted molecular weight of 24.4 kDa. Datta et al. (1999) developed transgenic rice plants constitutively expressing TLP (D34). Bioassays of transgenic rice plants challenged with *R. solani,* indicated that overexpression of TLP resulted in enhanced resistance compared to control plants. In order to purify and to characterize the rice TLP, we expressed the TLP gene D34 obtained from the cDNA library of rice (Velazhahan et al., 1998) in a baculovirus expression system.

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Materials and Methods

Expression of TLP in baculovirus-infected insect cells

The full-length rice TLP cDNA (D34) (Velazhahan et al., 1998) fragment was excised from pBS (TLP-D34) by enzymatic digestion with restriction endonuclease EcoRI. The 1049- bp nucleotide fragment containing the protein-coding region and 307-bp of the 3' untranslated region from TGA site to the EcoRI site was cloned in to the baculovirus expression vector, pVL1393 (Pharmingen, USA) under the control of the polyhedrin promoter and was named pVL1393 (TLP-D34). *Spodoptera frugiperda* (sf21) insect cells were grown as monolayer cultures in SF-900 II serum-free insect cell culture medium obtained from GIBCO-BRL (Gaithersburg, MD, USA) at 28 °C. *Spodoptera frugiperda* (sf21) cells were co-transfected with the constructed expression plasmid, pVL1393 (TLP-D34) and the AcMNPV DNA (BaculoGold, Pharmingen) according to the manufacturer's instructions. Recombinant baculovirus containing the rice TLP gene were amplified and used for expression studies. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Blue staining and western blot analysis were used to monitor the production of TLP.

Western blot analysis

Sf21 cell cultures (50 µl) were removed and centrifuged at 12,000g for 5 min and the supernatant was removed. Cells were suspended in 50 µl of 2x SDS-gel loading buffer [4% SDS, 125 mM Tris-HCl pH 6.7, 30% (v/v) glycerol, 0.002% (w/v) bromophenol blue and 2% (v/v) ß-mercaptoethanol] and subjected to 12% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Mighty Small II unit (Hoefer, California, USA) according to the method of Laemmli (1970). After electrophoresis, the proteins were transferred to a PVDF membrane using a Bio-Rad semi dry transfer cell (Bio-Rad, USA). The membrane was then blocked in Tris-buffered saline containing 0.05% (v/v) Tween-20 supplemented with 2.5% (w/v) gelatin. Antiserum raised against a bean TLP (kindly provided by Dr. O. P. Sehgal, University of Missouri, Columbia, MO, USA) was used as primary antibody at 1:1000 dilution. Detection of TLP on the membrane was performed according to Winston et al. (1987) using a 1:1000 dilution of horseradish peroxidase conjugated goat-anti rabbit IgG (Bio-Rad, USA). Color development was with 4-chloro-1-naphthol (Bio-Rad, USA).

N-terminal sequencing

For N-terminal sequencing of the expressed protein the crude proteins from insect cells was subjected to 12% SDS-PAGE and then they were electroblotted onto PVDF (Immobilon-P) membrane using a Bio-Rad semi-dry transblot unit (Bio-Rad, USA). The protein band detected by TLP antiserum was subjected to automated Edman degradation using an Applied Biosystems Sequencer at the Biotechnology Microchemical Core Facility, Kansas State University, Manhattan, Kansas, USA. Database search was performed with the BLAST 2.0 program from the National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD World Wide Web server.

Results and Discussion

The baculovirus expression system has been widely used for the expression of a variety of foreign proteins in insect cells (Luckow and Summers, 1988; Gopalakrishnan et al., 1995) including several plant proteins (Andrews et al., 1988; Bustos et al., 1988; Vernet et al., 1990; Nagai et al., 1992; Macdonald et al., 1994; Thompson et al., 1994). One of the major advantages of BEVS is that it can be used to produce relatively large quantities of post-translationally modified heterologous proteins. The results of the present study indicated that the TLP (23 kDa) was expressed abundantly and localized in the insect cells and not in the culture medium *(Figures 1, 2).* The N-terminal amino acid sequence of the 23 kDa rice TLP has 78% identity with oat TLP, 76% identity with thaumatin from *Thaumatococcus daniellii,* 73% identities with barley, maize and grapes TLPs and 68% identity with tobacco TLP. The N-terminal sequence of the 23-kDa protein with the sequences of mature thaumatin and other TLPs in the GenBank database by Blast search, indicates that they all have N-termini closely related to the sequence ATFAITN found for the TLP expressed in insect cells *(Table 1).* Thus the TLP expressed in insect cells is processed correctly as in plant cells. But the protein is not secreted into the medium indicating some differences between plant and insect cell secretion pathways. The production of high levels of recombinant protein with the strong baculovirus polyhedrin promoter and the shut down of host protein synthesis following baculovirus infection might have led to a limitation in the supply of secretory assistance factors (Jarvis and Garcia, 1994).

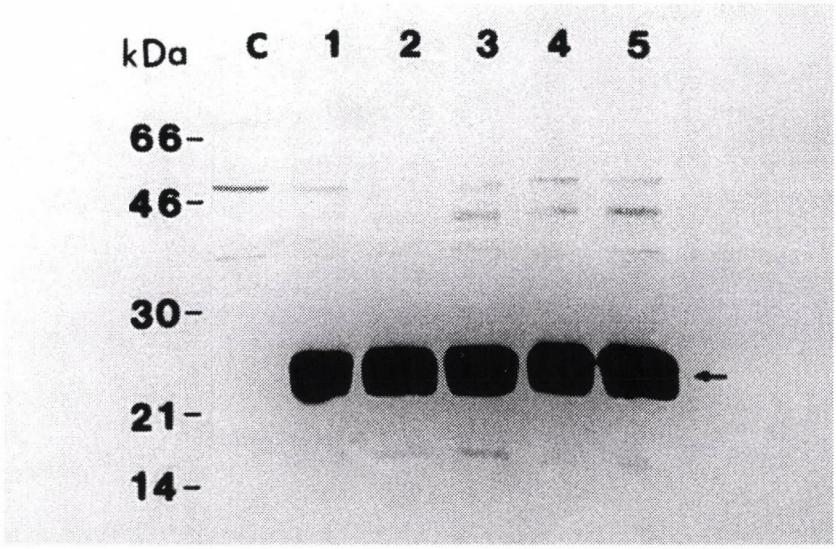


Fig. 1. Western blot analysis of TLP expressed in *Spodoptera frugiperda* (sf21) cells. C, total proteins from uninfected sf21 cells, Lanes 1–5, total proteins from sf21 cells infected with recombinant baculovirus containing the rice TLP gene. Position of size markers is indicated on the left

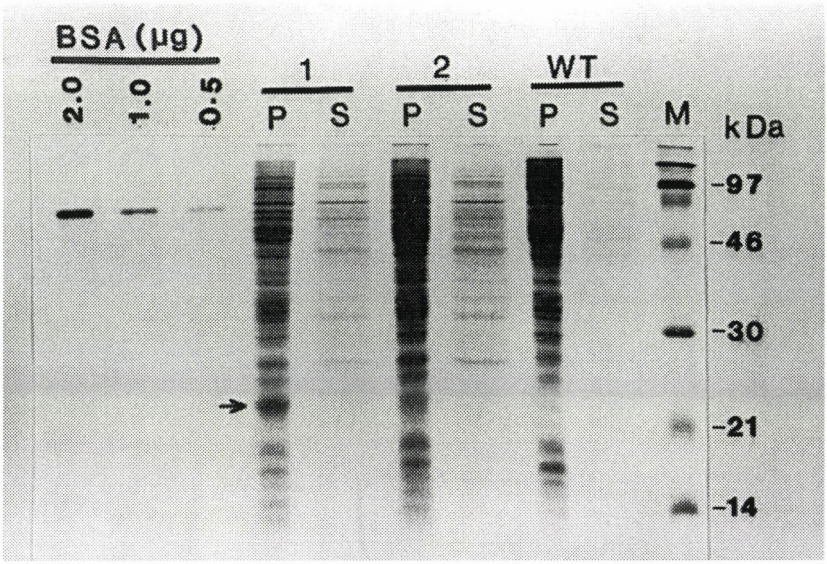


Fig. 2. Coomassie blue stained SDS-PAGE gel showing expression of TLP in *Spodoptera frugiperda* (sf21) cells. Position of recombinant protein is indicated by arrow. P, cell pellet; S, supernatant; WT, wild type cells

Table 1

Alignment of the N-terminal amino acid sequence of the 23-kDa rice TLP with TLPs of other plants in the database

A	T	F	A	I	T	N	R	C	Q	Y	T	V	W	P	A	A	V	P	Rice TLP D34
A	T	F	T	I	T	N	N	C	G	Y	T	V	W	P	A	A	I	P	Oat
A	T	F	T	V	I	N	K	C	Q	Y	T	V	W	A	A	A	V	P	Barley
A	T	F	T	I	T	N	N	C	G	F	T	V	W	P	A	A	T	P	Maize
A	T	F	N	I	K	N	N	C	P	Y	T	V	W	P	A	A	T	P	Wheat
A	T	F	N	I	Q	N	H	C	S	Y	T	V	W	A	A	A	V	P	Grape
A	T	F	E	I	V	N	R	C	S	Y	T	V	W	A	A	A			Thaumatin
A	T	F	D	I	V	N	Q	C	T	Y	T	V	W	A	A	A	S	P	Tobacco

The N-terminal amino acid sequence of the 23-kDa rice TLP was compared with the TLP sequences of *Avena sativa* (GenBank accession No. L39777), barley (GenBank accession No. S17684), maize (GenBank accession No.U82201), wheat (GenBank accession No. X97687), grape (GenBank accession No. Y10992), thaumatin from *Thaumatococcus daniellii* (GenBank accession No. P02883) and tobacco (GenBank accession No. P07052).

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Reactions of Promising Sorghum Genotypes to a Distinct Strain of Sorghum Mosaic Potyvirus (SrMV-IBS) Prevalent in Peninsular India

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Seventy-one promising sorghum genotypes were scored for their resistant status to SrMV-IBS using their disease severity reactions (DSR), infectivity levels (IL) and virus contents as inferred from virus absorbance values (AbV). Based on IL, 9% sorghum genotypes were rated as less susceptible (LS), 24% as moderately susceptible (MS) and 67% as highly susceptible (HS) to SrMV-IBS. Based on DSR, 20% sorghum genotypes were rated as LS, 28% as MS, and 51% as HS to SrMV-IBS. Based on virus contents (AbV), a symptomless IS-7999 was rated as tolerant (T), 3% as LS, 35% as MS and 61% as HS to SrMV-IBS. None of the sorghum genotype showed hypersensitive type resistance (HR) or immunity (I) to SrMV-IBS. Present virus isolate (SrMV-IBS-Tx2786) infected all the 43-sorghum genotypes found resistant or tolerant to a SrMV-IBS-CK-60A sorghum isolate. Combination of IL, DSR and AbV criteria was found more descriminate in scoring level of resistance of sorghum genotypes to SrMV-IBS isolates than a single criterion (DSR) used earlier.

Keywords: resistance criteria, sorghum genotypes, SrMV-IBS.

Recently sugarcane mosaic virus (SCMV) complex comprising various SCMV (A, B, D, E, H, I, M, Sc, BC, Sabi) and maize dwarf mosaic virus (MDMV-A, B, D, E, F, O) strains (Toler, 1985) have been separated into four distinct potyviruses including SCMV itself (A, B, D, E, MDB, Sc, BC, Sabi), sorghum mosaic potyvirus, SrMV (SCMV-H, I, M), MDMV (A, D, E, F) and Johnsongrass mosaic potyvirus, JGMV (Australian SCMV-Jg, USA MDMV-O, KS1) based on amino acid sequences, amino terminal serology, reactions of differential sorghum and oat cultivars, profiling of coat proteins, serology and morphology of cytoplasmic cylindrical inclusions (CCI) and molecular hybridization with probes corresponding to 3' non-coding region (Ford et al., 1989; Frenkel et al., 1992; Lesemann et al., 1992; McKern et al., 1991; Shukla et al., 1989; Shukla and Teakle, 1989; Teakle et al., 1989; Tosic et al., 1990).

A sugarcane potyvirus (SCPV) naturally infecting a multiple virus (SCMV-H, MDMV-D, MDMV-E, SCMV-MDB) resistant sorghum line, Tx2786 (Toler, 1980) and sugarcane (cv. Co.740) in Marathwada region of Maharashtra State, India was identified as an immunity (QL3) breaking strain of SrMV (SrMV-IBS) based on particle and CCI morphology, serological and biological properties (Mali, 1995; 1996; 1999). SrMV-IBS was also found widely prevalent in sugarcane growing districts of Maharashtra, Andhra Pradesh and Tamil Nadu States (peninsular India) (Mali and Thakur, 2000). The virus is

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spread down wind naturally from sugarcane to sorghum crops grown in the proximity of sugarcane via aphid vectors and infects Krish sorghums (QL3, QL11) carrying resistance to other SrMV strains (H,I,M) (Giorda et al., 1986; Grisham, 1994; Mali and Garud, 1994; Mali and Thakur, 1999).

The virus is an economically important pathogen on sorghum in India. Hence its management is of utmost importance in order to mitigate the losses caused by this virus in sorghum. One of the best method to control this virus is through the use of resistant cultivars (Mali, 1992). Earlier, 4% (70/1811) resistant sorghum genotypes to a CK-60A isolate of SrMV-IBS were identified using DSR criteria (Garud and Mali, 1980; 1981). Subsequently sorghum genotypes resistant to one isolate were found susceptible to other isolate (Mali, 1994). Therefore, present investigations were made to screen these 4% sorghum genotypes having resistant to CK-60A virus isolate against a more aggressive virus isolate (Tx2786 sorghum isolate) of SrMV-IBS by combining three criteria (IL, DSR, AbV) for scoring the test sorghum genotypes for their resistant status to both of these virus isolates (CK-60A and Tx2786 sorghum isolates).

Materials and Methods

Mechanical transmission

The SrMV-IBS originally isolated from a virus infected sugarcane (*Saccharum officiarum* cv. Co.740) grown at Parbhani (Marathwada region, Maharashtra State) was mechanically transmitted to and maintained in an insect free glasshouse on a multiple virus resistant sorghum inbred line, Tx2786 *(Sorghum bicolor)* (obtained from R. W. Toler, Texas A and M University, College Station, Texas, USA) was used as virus inoculum source in mechanical inoculation of 71-promising sorghum genotypes *(Table 1a, b)* (originating from ICRISAT germplasm source) in glasshouse at 13 to 30 °C. Virus inoculum was prepared by grinding symptomatic leaf tissue with a pestle and morter in the presence of a cold, neutral 0.05M potassium phosphate buffer containing 0.075% thioglycerol (PBT) in a 1: 9 (w/v) proportion. Twenty plants of each sorghum genotypes were used. The plants were inoculated at the 3-leaf stage by conventional leaf rub method. Carborundum (800 mesh) was used as an abrasive. Observations on IL and DSR were recorded 4 to 6 weeks after virus inoculations. The plants that did not show symptoms after a period of 6 weeks were back indexed on Tx2786 sorghum test plants for detecting latent infections if any. The DSR of sorghum genotypes was scored on a 0 to 5 scale where 0 = immune or symptomless, 1 = local lesions only, 2 = mild mosaic (MM), 3 = strong mosaic (SM), 4 = red stripe (RS) / red leaf (RL) with slight necrosis (n) and 5 = severe RS / RL with general necrosis (N).

Serological assays

Serological assay was accomplished using penicillinase based direct antigen coating form of enzyme-linked immunosorbent assay (PNC-DAC-ELISA) and polyclonal

antiserum of SrMV-IBS (produced in rabbits at ICRISAT). The procedure followed for DAC-ELISA was essentially the same as described by Hobbs et al. (1987); Sudarshana and Reddy (1989). Plant extracts were prepared in a coating buffer (0.05M sodium carbonate buffer, pH 9.6) at 10^{-2} dilutions. Unfractionated (whole) antiserum cross-absorbed with healthy sorghum (Tx2786) leaf extracts (in 1:20 proportions) was diluted to 1:1000 in antibody buffer, PBS-Tween 20-PVP (0.02M potassium phosphate, pH 7.3, 0.15M NaCl, 0.003M KCl, 0.05% Tween-20, 2% polyvinyl-pyrrolidone). Enzyme conjugate (penicillinase linked anti-rabbit IgG in goat) was diluted to 1:2500 in conjugate buffer (PBS-Tween 20-PVP-ovalbumin). After adding bromothymol blue (BTB) mixture, the reactions were observed for 30 to 60 minutes and the results were quantitated by measuring a loss of BTB at A_{620nm} using an ELISA microplate reader (Multiskan Plus Version, Labsystems). The positive (virus) and healthy (sorghum leaf tissue extracts) controls were also included.

Results and Discussion

Of the 71-sorghum genotypes screened *(Table 1a, b),* the SrMV-IBS caused definitive systemic symptoms in 70-genotypes and symptomless infections in a single sorghum genotype (IS-7999). Amongst symptomatic 70-genotypes, the IL, DSR and AbV were found to vary between sorghum genotypes.

Infectivity levels (IL)

The IL of Tx2786 isolate of SrMV-IBS was found to vary between 70-symptomatic sorghum genotypes and ranged from 10 to 100%. The lowest IL (10%) was recorded by a single genotype (IS 4243) and highest (100%) by 17(24%) sorghum genotypes *(Table 1a, b).* Comparatively low IL (40 to 44%) were also recorded by 7% sorghum genotypes. Intermediate IL (50 to 70%) were recorded by 20% and high IL (80 to 100%) by 64% sorghum genotypes. On the basis of IL, 6% sorghum genotypes (recording IL between 10 to 44%) were rated as less susceptible (LS), 29% (recording IL between 50 to 70%) as MS, and 64% (recording IL between 80 to 100%) as HS to SrMV-IBS *(Table 1a, b).* Earlier, the IL of the same isolate (Tx2786) of SrMV-IBS was also observed to vary between 18-symptomatic differential sorghum inbred lines and ranged from 29 to 100%. It was low (IL: 29–40%) in 11%, medium (IL: 53–87%) in 56% and high (IL: 100%) in 33% sorghum differentials tested (Mali and Thakur, 1999). These results also indicated that 25 (out of 43) sorghum genotypes which were symptomless carriers of CK-60A isolate (Garud and Mali, 1980; 1981) of SrMV-IBS (Mali, 1994), expressed definitive systemic symptoms after sap-inoculation with Tx2786 virus isolate. It also infected remaining 18 sorghum genotypes which were resistant to CK-60A isolate *(Table 3).* Thus, Tx2786 isolate was more aggressive type compared to CK-60A isolate. The variation in IL between 18-symptomatic differential sorghum inbred lines for these two isolates (CK-60A and Tx2786) of SrMV-IBS have also been observed earlier (Mali, 2000; Mali and Thakur, 1999) thereby indicating their distinctness in aggressivity to sorghum.

Table 1a

Sorghum genotypes reacting with mosaic symptoms to SrMV-IBS/Tx2786 isolate upon sap transmission in the glasshouse

Sorghum genotypes	Infectivity (% IL)*	Symptoms DSR**	DAC-ELISA/SrMV-IBS	
			Reactions	AbV
1. IS-1087	90.0	2	(+)[a]	0.48
2. IS-9093	90.0	2	(+)	0.49
3. IS-8150	100	2	+	0.85
4. IS-6885	100	2	+	0.85
5. IS-9069	90.0	2	+	0.87
6. IS-9668	86.0	2	+	0.92
7. IS-9066	86.0	2	+	0.92
8. IS-5169	90.0	2	+	1.02
9. IS-9676	100	2	+	1.04
10. IS-5099	100	2	+	1.06
11. IS-8721	70.0	2	+	1.06
12. IS-5769	40.0	2	+	1.06
13. IS-2570	100	2	+	1.12
14. IS-4243	10.0	2	+	1.28
15. IS-9287	60.0	3	+	0.55
16. IS-73	80.0	3	+	0.71
17. IS-2301	70.0	3	+	0.73
18. IS-844	90.0	3	+	0.74
19. IS-2601	60.0	3	+	0.85
20. IS-5080	70.0	3	+	0.86
21. IS-8672	50.0	3	+	0.87
22. IS-5401	80.0	3	+	0.96
23. IS-8671	90.0	3	+	0.96
24. IS-3507	100	3	+	1.03
25. IS-115	100	3	+	1.03
26. IS-110	100	3	+	1.04
27. IS-10297	100	3	+	1.06
28. IS-8722	70.0	3	+	1.06
29. IS-5790	90.0	3	+	1.08
30. IS-9609	50.0	3	+	1.08
31. IS-7499	100	3	+	1.09
32. IS-9805	80.0	3	+	1.13
33. IS-4710	40.0	3	+	1.22
34. IS-4870	60.0	3	+	1.28
35. IS-7999	0	0	+	0.28
36. Tx2786				
a. +ve control	100	3	+	1.30
b. H-control	0	0	–	0.05

[a]Weak reactions in parenthesis indicate the AbV being recorded is < 0.50

*Infectivity levels (IL) based on total of 20 plants of each sorghum genotype

**Disease severity reactions (DSR): 0 = symptomless, 2 = mild mosaic, 3 = strong mosaic

Table 1b

Sorghum genotypes reacting with red stripe, red leaf and necrotic symptoms to SrMV-IBS/Tx2786 isolate upon sap transmission in the glasshouse

Sorghum genotypes	Infectivity (% IL)*	Symptoms DSR**	DAC-ELISA/SrMV-IBS	
			Reactions	AbV
1. IS-635	90.0	4	+	0.64
2. IS-413	84.0	4	+	0.67
3. IS-2309	60.0	4	+	0.68
4. IS-9617	80.0	4	+	0.75
5. IS-2740	80.0	4	+	0.76
6. IS-5770	50.0	4	+	0.85
7. IS-410	86.0	4	+	0.94
8. IS-7035	90.0	4	+	0.99
9. IS-9606	80.0	4	+	1.04
10. IS-7122	70.0	4	+	1.05
11. IS-9736	90.0	4	+	1.05
12. IS-4758	80.0	4	+	1.05
13. IS-2522	100	4	+	1.06
14. IS-8354	90.0	4 (RL/n)	+	1.09
15. IS-952	100	4 (RL/n)	+	1.10
16. IS-4416	60.0	4	+	1.11
17. IS-4909	90.0	4	+	1.14
18. IS-7998	80.0	4	+	1.15
19. IS-8707	100	4 (RS/RL/n)	+	1.17
20. IS-5096	100	4	+	1.18
21. IS-9866	100	4	+	1.19
22. IS-4550	40.0	4	+	1.28
23. IS-2803	90.0	4	+	1.28
24. IS-5007	70.0	4	+	1.29
25. IS-10734	70.0	4	+	1.34
26. IS-3943	80.0	4	+	1.64
27. IS –9113	80.0	5	+	0.70
28. IS-5282	80.0	5	+	0.95
29. IS-3155	80.0	5 (RS/RL/N)	+	0.98
30. IS-9677	70.0	5	+	1.08
31. CS-3541	68.0	5 (RS/RL/N)	+	1.12
32. IS-4996	44.0	5	+	1.15
33. IS-9409	40.0	5 (RS/RL/N)	+	1.15
34. IS-1518	100	5 (RS/RL/N)	+	1.17
35. CK-60A	88.0	5 (RS/RL/N)	+	1.18
36. IS-4731	90.0	5	+	1.23
37. Tx2786				
a. +ve control	100	3	+	1.30
b. H-control	0	0	–	0.05

*Infectivity levels (IL) based on total of 20 plants of each test sorghum genotype

**Disease severity reactions (DSR): 0 = symptomless,
4 = red stripe (RS) and/or red leaf (RL) and slight necrosis (n), 5 = RS/RL and general necrosis (N)

Disease severity reactions (DSR)

Amongst 70-symptomatic sorghum genotypes, the Tx2786 isolate of SrMV-IBS caused DSR = 2 type symptoms in 20%, DSR = 3 type in 28%, DSR = 4 type in 37% and DSR = 5 type in 4% sorghum genotypes *(Table 1a, b)*. Amongst DSR = 4 and 5 types, the present virus isolate caused only RL in 2 (3%), both RL and RS in 6 (9%) sorghum genotypes. In 70-symptomatic sorghum genotypes, the DSR = 4 types were higher (37%) compared to DSR = 3 (28%), DSR = 2 (20%) and DSR = 5 (14%) types. On the basis of DSR, 20% sorghum genotypes with DSR = 2 were rated as LS, 28% with DSR = 3 as MS and 51% with DSR = 4 and 5 as HS to Tx2786 isolate of SrMV-IBS *(Table 1a, b)*. Earlier, the DSR caused by the same isolate (Tx2786) of SrMV-IBS was also observed to vary between 18-symptomatic sorghum differentials. This virus isolate caused DSR = 2 type symptoms in 22%, DSR = 3 type in 56% and DSR = 4 and 5 types in 22% sorghum differentials tested (Mali and Thakur, 1999).

It has been reported that the sorghum genotypes that produces only mosaic reactions to potyviruses within SCMV subgroup (MDMV, JGMV, SCMV-MDB) are tolerant (maximum yield loss: 0 to 30%) compared to those producing RS/N (JGMV) (maximum yield loss: 60%) or RL/N (MDMV, SCMV-MDB) (maximum yield loss: 10 to 90%) reactions (Dean and Coleman, 1959; Henzell et al., 1979; Penrose, 1974; Seifers and Hackerott, 1987; Teakle et al., 1970; Toler, 1980). Similar trend has been observed earlier (Mali and Garud, 1994) for SrMV-IBS in 18-differential sorghum inbred lines tested under field conditions. Sorghum differentials with mosaic reactions sustained less yield losses (8 to 13%) compared to those reacting with RS/N (maximum yield loss of 41%) (Mali and Garud, 1994). According to this criterion, 51% sorghum genotypes expressing RS/RL/N reactions could be rated as susceptible and remaining 49% sorghum genotypes expressing MM/SM rated as tolerant to Tx2786 isolate of SrMV-IBS. However, this type of scoring seemed apparently inappropriate and much accurate genotypes scoring using 0 to 5 scale as proposed by Toler (1980) for MDMV was adapted for accomodating various reactions expressed by virus (SrMV-IBS) inoculated sorghum genotypes including those carrying virus without producing any obvious symptoms.

Earlier, CK-60A sorghum genotype was found to react with both RS and RL to CK-60A virus isolate whereas CS-3541 sorghum genotype reacted with SM to the same virus isolate. In case of Tx2786 virus isolate, the trend was reverse at least in case of CS-3541 sorghum genotype invariably reacting with RS/RL *(Table 1b)* but not SM alone to Tx2786 isolate as was the case with CK-60A virus isolate (Garud and Mali, 1980; 1981). Thus CS-3541 was identified as a sorghum differential cultivar for separating these two virus isolates when they occurred in mixed infections. Recently, CK-60A, CS-3541 and Tx2786 isolates have been identified as distinct pathotypes of SrMV-IBS based on their percentage of necrotic reactions (RS / RL) elicited in 18-differential sorghum inbred lines (Mali, 2000).

Virus contents of resistant and susceptible sorghum genotypes (AbV)

The test sorghum genotypes were also found to vary in virus contents (as inferred from virus absorbance ELISA values, AbV) upon sap inoculations with Tx2786 isolate of

SrMV-IBS *(Table 1a, b).* The lowest AbV (0.28) was recorded in IS-7999 symptomless sorghum genotype. Thus, it was rated as tolerant (T) to SrMV-IBS. It was also rated as T on the basis of IL and DSR. Additionally, two sorghum genotypes (IS-1087, IS-9093) recording AbV between 0.48 to 0.49 were also rated as virus tolerant (T) or LS genotypes as these two genotypes recorded AbV < 0.50 which is referred to as a minimum AbV for scoring DAC-ELISA samples as positive for SrMV-IBS (Mali, 1994). Besides this, 35% sorghum genotypes recording AbV between 0.55 to 0.99 were rated as MS and 61% sorghum genotypes recording AbV between 1.02 to 1.64 as HS to Tx2786 isolate of SrMV-IBS. Earlier, variations in virus contents of 18-differential sorghum inbred lines were also observed upon sap inoculation with Tx2786 isolate of SrMV-IBS in the glasshouse (Mali and Thakur, 1999). On the basis of AbV recorded in mosaic expressing sorghum genotypes *(Table 1a),* 50% genotypes were highly susceptible to this virus isolate. Thus, just to score sorghum genotypes as virus resistant / tolerant based on mosaic reactions was found inappropriate on the basis of virus contents Earlier, Seifers and Caceres (1988) have also used ELISA to determine virus (SCMV-MDB, syn. MDMV-B) titres in resistant and susceptible sorghum genotypes. They recommend virus titre analysis of sorghum genotypes in screening work involving SCMV-MDB since resistance to MDMV had been correlated with low virus titres in MDMV-infected corn (Tu and Ford, 1970) and with barley yellow dwarf virus (BYDV) -infected cereals (Jedlinski et al., 1977; Skaria et al., 1985). Using the titre criterion, three sorghum genotypes (IS-7999, IS-1087, IS-9093) could be considered resistant to SrMV-IBS in the present work.

Correlations between IL, DSR and virus contents (AbV)

Three criteria (IL, DSR, AbV) used in scoring sorghum genotypes as resistant or susceptible to SrMV-IBS *(Table 2)* indicated low correlations (as worked out on percentage basis) between these three parameters. It was to the extent of 28% between IL, DSR and AbV; 21% between DSR and AbV; 20% between IL and AbV and 18% between IL ans DSR. Similar trend was found by us earlier when these three criteria were used to score 18-differential sorghum inbred lines as resistant or susceptible to SrMV-IBS (Mali and Thakur, 1999).

Seifers and Caceres (1988) have reported high correlations between titres of SCMV-MDB (syn. MDMV-B) determined in two sorghum genotypes (Colt, Bugoff) by ELISA and infectivity assay. They recommend to combine infectivity tests with ELISA in screening work in order to ensure that infective virus and not just coat protein is being measured. On the other hand, Stoddard et al. (1987) observed low level of correlations between the ELISA and symptomatology used to evaluate wheat germ plasm for resistance to wheat streak mosaic virus (WSMV). According to them, the observations of symptom expressions in an assay for viral diseases should not be used exclusively because as with many viral diseases, the degree of symptom severity is not always proportional to viral replication within the host. As a result, making use of assays that determine the viral content (by ELISA) in addition to symptomatology assays provide an accurate method of screening wheat lines and germ plasm for WSMV resistance. This also holds good for the low level of correlation observed between AbV (ELISA values expressing virus content)

Table 2

Sorghum genotypes showing positive correlations with respect to IL, DSR and AbV

IL/DSR/AbV*	DSR/AbV	IL/AbV	IL/DSR
IS-9606	IS-1080	IS-5169	IS-5759
IS-9736	IS-9093	IS-9676	IS-8722
IS-4758	IS-73	IS-5099	IS-9609
IS-2522	IS-844	IS-2570	IS-4870
IS-8354	IS-5404	IS-4243	IS-635
IS-952	IS-8671	IS-3507	IS-413
IS-4909	IS-7122	IS-115	IS-9617
IS-7998	IS-4416	IS-110	IS-2740
IS-8706	IS-4550	IS-10297	IS-410
IS-5096	IS-5007	IS-5790	IS-7035
IS-9866	IS-10734	IS-7499	IS-9113
IS-3943	IS-9677	IS-9805	IS-5282
IS-1518	CS-3541	IS-2309	IS-3155
CK-60A	IS-4996	IS-5770	
IS-4731	IS-9409	(Tx2786)	
IS-9287			
IS-2301			
IS-2601			
IS-5080			
IS-8672			
Total = 71	71	71	71
With positive correlations = 20	15	14	13
Correlations (%) = 28	21	20	18

*IL = infectivity levels, DSR = disease severity reactions,
AbV = virus absorbance values at A_{620nm} in PNC-DAC-ELISA

and the symptom severity (DSR) in the present work. As advocated by others (Seifers and Caceres, 1988; Stoddard et al., 1987), the combinations of these three criteria (IL, DSR, AbV) was also found more descriminating in scoring sorghum genotypes as susceptible or resistant to SrMV-IBS in spite of the fact that the correlation between these parameters found was also low in the present work.

Differential reactions of sorghum genotypes to SrMV-IBS isolates

Comparative reactions of two isolates, CK-60A and Tx2786 of SrMV-IBS as given in *Table 3* indicates that all the 25-symptomless carrier sorghum genotypes (DSR = 0) rated as T (tolerant) to CK-60A virus isolate were susceptible to Tx2786 isolate. The remaining 18-sorghum genotypes rated as immune (I) to CK-60A isolate were also infected by Tx2786 isolate of SrMV-IBS (Garud and Mali, 1980; 1981). The Tx2786 virus isolate caused definitive systemic symptoms in these 43-resistant genotypes to CK-60A virus isolate including DSR = 3 types in 47% and DSR = 4 type in 51% sorghum geno-

Table 3

Comparative reactions of 43-sorghum genotypes to two isolates (CK-60A and Tx2786) of SrMV-IBS

Sorghum genotypes	Tx2786 isolate Ratings*/DSR**	CK-60A isolate Ratings/DSR
IS-115	S / 3	T / 0
IS-1087	S / 3	T / 0
IS-4243	S / 3	T / 0
IS-4710	S / 3	T / 0
IS-4731	S / 4	T / 0
IS-4909	S / 4	T / 0
IS-4996	S / 4	T / 0
IS-5080	S / 3	T / 0
IS-5096	S / 4	T / 0
IS-5099	S / 3	T / 0
IS-5282	S / 4	T / 0
IS-5401	S / 3	T / 0
IS-6885	S / 3	T / 0
IS-7035	S / 4	T / 0
IS-7122	S / 4	T / 0
IS-8150	S / 3	T / 0
IS-8671	S / 3	T / 0
IS-8706	S / 4	T / 0
IS-9069	S / 3	T / 0
IS-9113	S / 4	T / 0
IS-9409	S / 4	T / 0
IS-9609	S / 4	T / 0
IS-9666	S / 3	T / 0
IS-10297	S / 3	T / 0
IS-10734	S / 4	T / 0
IS-73	S / 3	I / 0
IS-844	S / 3	I / 0
IS-952	S / 4	I / 0
IS-1518	S / 4	I / 0
IS-2309	S / 4	I / 0
IS-2507	S / 3	I / 0
IS-2522	S / 4	I / 0
IS-2601	S / 3	I / 0
IS-2740	S / 4	I / 0
IS-2803	S / 4	I / 0
IS-3155	S / 4	I / 0
IS-4758	S / 4	I / 0
IS-5007	S / 4	I / 0
IS-5169	S / 3	I / 0
IS-7499	S / 3	I / 0
IS-8722	S / 3	I / 0
IS-9736	S / 4	I / 0
IS-9805	S / 3	I / 0

*S = susceptible, T = tolerant, I = immune

**Disease severity reactions (DSR): 0 = symptomless, 3 = strong mosaic, 4 = red stripe, red leaf and slight necrosis (RS/RL/n)

types. On the basis of aggressiveness and virulence to 18-differential sorghum inbred lines, these two virus isolates, CK-60A and Tx2786 have recently been considered as distinct pathotypes of SrMV-IBS (Mali, 2000; Mali and Thakur, 1999) besides being serologically found indistinguishable (Mali, 1994). These results indicate that the resistance in sorghum to SrMV-IBS is isolate specific as the case reported for the BYDV with barley and oat host combinations (Skaria et al., 1985).

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Some Aspects of Pepper Virus Research

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Pepper (*Capsicum annuum* L.) is one of the most important national product of Hungary. The success of production is greatly influenced by different pathogens. Among them viruses may cause considerable yield losses, depending on environmental factors, conditional stage of the plants and resistance level of the variety. This paper gives a short overview about Hungarian pepper virus research with an outlook to the international situations. Results of 25 years old surveys of virus diseases in forced and field conditions, pot experiments about the interaction of different pathogens, resistance level of pepper varieties, sources of resistance and breeding strategies for virus resistance are included in this review.

Keywords: pepper, viruses, resistance, breeding.

Last decades vegetable growing in glasshouses and plastic tunnels achieved considerable results in Hungary. Vegetable growing area is about 80 ha of glasshouses and 3000–4000 ha of plastic tunnels. Pepper occupies the first place concerning surface under both of glasshouse and plastic tunnels (Csikai, 1996). The consumption of sweet pepper increases both in Hungary and abroad. Changing of the eating habits create good export possibilities for Hungarian primeur pepper. Additionally, the red spice pepper is also important national product. Since 1996, an integrated pest management (IPM) program has been started in pepper growing, under cover (Budai et al., 1996).

The number of the viruses, which infect pepper under natural conditions is about 45 (Horváth, 1981, 1986a; Green and Kalloo, 1994; Edwardson and Christie, 1997; Gáborjányi et al., 1998a, b). Additionally, other viruses can infect it under artificial conditions. Out of the 45 viruses only six [tobacco mosaic *tobamovirus* (TMV), tomato mosaic *tobamovirus* (ToMV), cucumber mosaic *cucumovirus* (CMV), alfalfa mosaic *alfamovirus* (AMV), potato Y *potyvirus* (PVY) and potato X *potexvirus* (PVX)] have economic importance in Hungary. First cucumber mosaic virus was identified more than 50 years ago (Szirmai, 1941), and later, 12 other viruses and a phytoplasma have been detected as natural pathogens. Recently two other viruses, pepper mild mottle *tobamovirus* (PMMoV) and tomato spotted wilt *tospovirus* (TSWV) mean new threat for pepper (Wetter et al., 1984; Avgelis, 1986; Betti et al., 1986, 1988; Alonso et al., 1989; Gáborjányi et al., 1995a, b; Kiss, 1996a; Jenser et al., 1996; Skorič et al., 1997; Gitaitis et al., 1998; Kálmán et al., 2000) *(Table 1)*. The known host range of TSWV is expanding to include new cultivated and weed species (Momol et al., 2000; Díaz-Pérez and Pappu, 2000).

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Table 1

First descriptions of pepper pathogen viruses and stolbur phytoplasma in Hungary (after Gáborjányi et al., 1998b modified)

Viruses	References
Cucumber mosaic *cucumovirus* (CMV)	Szirmai (1941)
Alfalfa mosaic *alfamovirus* (AMV)	Szirmai (1944)
Tobacco mosaic *tobamovirus* (TMV)	Szirmai (1952)
Potato X *potexvirus* (PVX)	Szirmai (1952)
Potato Y *potyvirus* (PVY)	Horváth (1967)
Tomato aspermy *cucumovirus* (TAV)	Beczner et al. (1979)
Broadbean wilt *fabavirus* (BBWV)	Salamon et al. (1980)
Tomato mosaic *tobamovirus* (ToMV) syn:	Csilléry et al. (1983)
Dulcamara yellow fleck *tobamovirus* (DYFV) syn.: tomato mosaic *tobamovirus* Ob strain (ToMV-Ob)	Salamon et al. (1987)
Tomato spotted wilt *tospovirus* (TSWV)	Gáborjányi et al. (1995a)
Pepper mild mottle *tobamovirus* (PMMoV)	Kiss (1996a), Kálmán et al. (2000)
Henbane mosaic *potyvirus* (HeMV)*	Gáborjányi et al. (1997)
Sowbane mosaic *sobemovirus* (SoMV)*	Gáborjányi et al. (1997)
Stolbur phytoplasma (SP)	Szirmai (1956)

*only serologically detected

Survey of virus infections of pepper in glasshouses and plastic tunnels

Surveys have been carried out in order to determine virus infection under glasshouse and tunnels for more than twenty years. They were made mainly on the central pepper growing area and the Southern part of Hungary. These studies have been stated that thirteen different viruses can infect forced pepper. The relative incidences of these viruses were different, depending on years and the resistance level of the varieties *(Table 2).* By the end of the vegetation period the extent of the infectedness varied between 20 and 60%, which caused 5–40% yield losses (Kiss, 1996a, b; Huszka et al., 1996).

Virus diseases of field grown pepper

In the last decade wilting, yellowing symptoms and in severe cases the death of the pepper plants occurred in the main pepper growing areas in Hungary. Yield losses varied between 10 and 30% depending on years and growing regions. Besides abiotical and growing technological reasons, the different plant pathogens (e.g. *Verticillium, Fusarium, Sclerotinia, Rhizoctonia, Macrophomina, Phaeoramularia, Leveilula* species as fungi; *Pseudomonas, Xanthomonas* species as bacteria, one phytoplasma and viruses are believed to play role in the forming of complex yellowing syndroms. The general incidence of virus infection was about 15–60%, but reached 100% at the end of the vegetation period (Gáborjányi et al., 1996, 1997).

The presence of cucumber mosaic, alfalfa mosaic, tomato, tobacco mosaic, potato X, potato Y, henbane and Chenopodium mosaic viruses were detected by serological methods from the leaf samples, collected from field peppers. Majority of the samples were

Table 2

Viruses infecting pepper under glasshouse and tunnels in Hungary since 1975
[after Kiss (1996a, b) modified]

Viruses	Importance*	Transmission
Tobacco mosaic *tobamovirus*	+++++	M, S
Tomato mosaic *tobamovirus* (syn: Dulcamara yellow fleck *tobamovirus*)	+++++	M, S
Cucumber mosaic *cucumovirus*	+++++	A, M, S
Potato Y *potyvirus*	++++	A, M
Alfalfa mosaic *alfamovirus*	++++	A, M, P, S
Tomato aspermy *cucumovirus*	+++	A, M, S
Broad bean wilt *fabavirus*	+++	A, M
Potato X *potexvirus*	+++	M, F, C
Pepper mild mottle *tobamovirus*	++	M, S
Tomato spotted wilt *tospovirus*	++	T, M
Pepper yellow vein mosaic *varicosavirus* (?)	+	F
Pepper yellows/golden mosaic disease	+	W

*+++++ widespread for ages, economically important even todays; ++++ widespread for ages, less important; +++ widespread for ages, no important today; ++ isolated recently, spreading; + only symptoms, identification is in progress; A, aphids; F, soil-borne fungi; M, mechanical; P, pollen; S, seed; T, thrips; W, whitefly; C, *Cuscuta* species

infected in a complex by three or more viruses. The relative incidence of these viruses was different *(Table 3)*. Between the two main serotypes of CMV (To and D) the severe To was the more frequent in the fields (Beczner and Burgyán, 1983).

Relatively warm winters and hot summers of last years favoured the spreading and overwintering of aphids, leafhoppers and whiteflies, which are the vectors of some viruses and phytoplasma diseases. Additionally to viruses, stolbur phytoplasma was also identified by PCR technique from the field pepper samples suggesting that it plays important role in pepper decline (Süle and Viczián, 1996; Viczián et al., 1998).

Table 3

Relative incidence of pepper pathogen viruses in fields
[after Gáborjányi et al. (1998b)]

Viruses	Relative incidence (%)
Cucumber mosaic *cucumovirus*	77
Alfalfa mosaic *alfamovirus*	83
Tobacco and tomato mosaic *tobamoviruses*+	27
Potato Y *potyvirus*	44
Potato X *potexvirus*	44
Henbane mosaic *potyvirus**	61
Sowbane mosaic *sobemovirus**	27

+TMV and ToMV cannot been distinguished by the antisera ToMV D/H,
*only serologically detected

Interaction of pepper pathogens

Viruses always occur in complex infections with other pathogens (Conti and Masenga, 1977). Investigations were carried out to study the effect of three pepper pathogens (CMV, PVY and *Macrophomina phaseolina*) on the development and nutrient uptake of pepper in pot experiments (Kazinczi et al., 1998a, b). Virus infection alone did not influence significantly the development of the plants. *M. phaseolina* alone reduced the fresh weight of the roots and the shoots, suggesting the disturbance of water uptake of

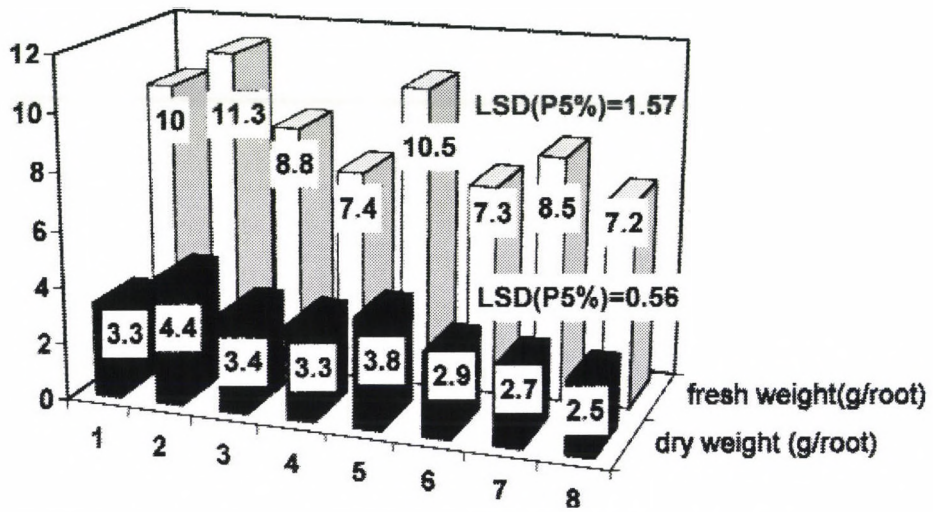


Fig. 1. The effect of different pathogens on the fresh and dry weight of the roots (1, Control; 2, CMV-U246; 3, PVY^{NTN}; 4, *M. phaseolina;* 5, CMV-U/246 + PVY^{NTN}; 6, CMV-U/246 + *M. phaseolina;* 7, PVY^{NTN} + *M. phaseolina;* 8, CMV-U/246 + PVY^{NTN} + *M. phaseolina)*

Table 4

The effect of different pathogens on the nitrogen (N), phosphorus (P) and potassium (K) content of pepper leaves

		Treatments*								
		1	2	3	4	5	6	7	8	$LSD_{P5\%}$
N	30th June	4.27	4.09	3.74	3.51	3.64	3.79	4.37	4.16	0.28
(%)	23th Aug	4.10	4.31	4.28	4.05	4.00	4.34	4.67	4.26	0.44
P	30th June	0.22	0.1	0.19	0.17	0.17	0.18	0.23	0.19	0.03
(%)	23th Aug	0.22	0.25	0.24	0.21	0.21	0.22	0.26	0.24	0.03
K	30th June	4.35	3.90	3.85	4.21	3.92	3.60	3.42	3.66	0.55
(%)	23th Aug	3.30	3.64	3.38	3.16	3.50	3.27	2.99	3.14	0.49

*1, Control; 2, CMV-U246; 3, PVY^{NTN}; 4, *M. phaseolina;* 5, CMV-U/246 + PVY^{NTN}; 6, CMV-U/246 + *M. phaseolina;* 7, PVY^{NTN} + *M. phaseolina;* 8, CMV-U/246 + PVY^{NTN}+*M. phaseolina*

roots due to the infection. However, several mixed infection of viruses and fungui drastically decreased the fresh and dry weight of the shoots and roots *(Figs 1, 2)*. Infections have played important role especially in the early nutrient content of the infected pepper leaves as compared to the healthy ones. In case of several mixed infection with viruses and *M. phaseolina* the reduction in the nutrient content of the pepper leaves was greater *(Table 4)*.

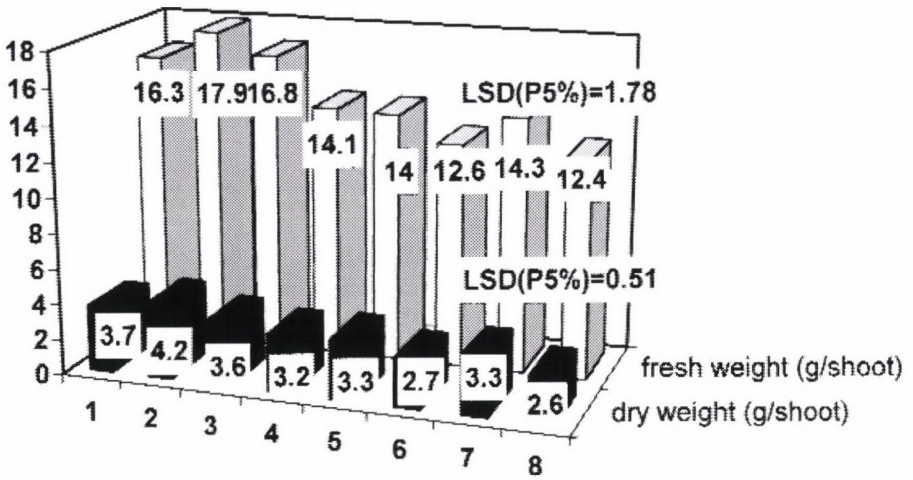


Fig. 2. The effect of different pathogens on the fresh and dry weight of the shoots (1, Control; 2, CMV-U246; 3, PVY^{NTN}; 4, *M. phaseolina;* 5, CMV-U/246 + PVY^{NTN}; 6, CMV-U/246 + *M. phaseolina;* 7, PVY^{NTN} + *M. phaseolina;* 8, CMV-U/246 + PVY^{NTN} + *M. phaseolina)*

Resistance of Hungarian pepper varieties to viruses

Before 1968 all the tested varieties in Hungary were susceptible to TMV, CMV, PVY and PVX infections (Horváth, 1967, 1969; Beczner and Horváth, 1969; Horváth, 1983) but after this time a breeding program was started to built the resistance genes into the different pepper varieties (Zatykó, 1982). In 1985 only 10%, but in 1995 31% of the registered Hungarian pepper varieties had some degree of virus resistance (Fehér and Kristóf, 1995; Fehér, 1996).

Leaf samples of about 40 Hungarian pepper varieties collected from fields were analysed by DAS-ELISA tests at the end of the vegetation period. According to this study the majority (65%) of the varieties were infected. Incidence of infections were the followings: CMV (22%), PVY (20%), AMV (10%) and PVX (8%). In the field test, only 6 out of the 40 varieties were free from viruses; 19 were infected by one virus; 11 by two viruses and 4 varieties suffered in a complex of three viruses (Gáborjányi et al., 1998a, b; 1999). Mixed infections, i.e. infection of a plant by more than one type of virus, occur commonly in nature and may result in a range of effects on the host as well as on the levels of accumulation and degrees of movement of either of the viruses involved. Those works

are also particularly interesting in which the restricted ability of one virus to move is alleviated by co-infection with another virus (Fuentes and Hamilton, 1991; Murphy and Kyle, 1995). Guerini and Murphy (1999) showed that in *C. annuum* cv. Avelar plants co-infected with PepMoV and CMV, PepMoV is able to enter, accumulate in and move within internal phloem, thereby allowing the virus to invade young tissues systemically.

Inoculations under glasshouse were made to evaluate the virus susceptibility of the varieties *(Table 5)* (Horváth et al., 2000a, b). Dabora F1, Hímes F1, Synthetic Cecei, Táltos Synthetic, Boni, Star, Cecil F1, Tizenegyes, Ciklon F1, Édes alma, Korona, Gigant F1, Alba Regia, Óriás Cseresznye varieties proved to be resistant to U-246 strain of CMV infection on the basis of symptoms, DAS ELISA and back inoculation. Results showed, that affinity of the different pepper varieties to the U-246 strain of CMV is not so strong. The majority of the tested varieties proved to be susceptible or at least tolerant to CMV (Zatykó, 1982; Fehér and Kristóf, 1995; Moór and Zatykó, 1995). Under field conditions, the extent of the resistance of pepper varieties to CMV varied from year to year, depending on ecological factors and phenological stage and condition of pepper plants (Tóbiás et al., 1978; Tóbiás and Molnár, 1983). It was demonstrated that the CMV resistance of pepper varieties at the seedling stage cannot always represent their resistance at the maturation stage. Generally speaking, resistance to CMV was enhanced from the seedling stage to the maturation stage more in pepper or chilli varieties than in sweet pepper varieties (Nono-Womdim et al., 1991; Wang et al., 1998). Resistance of local pepper lines was not influenced at temperatures above 30 °C (Stoimenova et al., 1998).

No varieties showing extreme resistance (immunity) have been found to TMV/U1 infection. Varieties: e.g. Keszthelyi Rezisztens, Dabora F1, Brill F1, Fehérözön Synthetic, Ciklon F1, Cecil F1 and Hímes F1 showed only local hypersensitive reaction to C/U1 strain of TMV infection. Plant resistance to TMV infection expressed in the form of restricted systemic movement of the virus has been described for many other host-virus relations (Nelson et al., 1993; Schaad and Carrington, 1996; Derrick and Barker, 1997; Guerini and Murphy, 1999). Systemic latent infection has been occurred in Rapires F1 variety. On the basis of symptomatology and DAS ELISA the variety was resistant, but during back inoculation local necrotic lesions and systemic mosaic symptoms were seen on 'Xanthi' and 'Samsun' plants, respectively, suggesting the presence of TMV in systemic leaves.

Eight varieties (Tuba, Fehérözön Synthetic, Boni, Alba Regia, Korona, Édes alma, Cecil F1 and Star) were found to be resistant to NTN strain of PVY. In spite the fact, that the new, NTN strain of PVY (which was described at first time in Hungary by Beczner et al., 1984) causes severe injuries on potato, only mild mosaic symptoms could be seen even on susceptible pepper varieties and low extinction values obtained from ELISA tests indicated that PVY^{NTN} occurs at low concentration in susceptible plants. Our preliminary results show that affinity of the different *Capsicum* genotypes to this resistance breaking strain of PVY is not so strong, as compared with *Solanum* species and accessions (Takács et al., 2000; Kazinczi et al., 2001).

Resistance to TSWV was established in three varieties (Alba Regia, Golden California and Tüzes Piros) (Gáborjányi et al., 1998b).

All of the tested pepper varieties showed resistance to SoMV. Neither the inoculated nor the non-inoculated leaves showed symptoms and the virus could not be detected by serological and biological tests. This virus previously had been serologically detected from seven varieties of field pepper (Gáborjányi et al., 1997). Until now isolation of SoMV from pepper has not yet managed. Natural occurrence of SoMV on *Chenopodium hybridum* was described from potato ecosystem (Horváth et al., 1993) and since *Chenopodium* species are also important weeds of field pepper, they may be reservoirs of SoMV and primary infection sources.

Among the examined varieties four (Boni, Korona, Édes alma, Star) were resistant to three viruses (SoMV, CMV-U/246 and PVY^{NTN}). Alba Regia was resistant to four viruses (SoMV, CMV-U/246, PVY^{NTN}, TSWV). One variety (Cecil F1) displayed complex, extreme resistance to SoMV, PVY^{NTN}, CMV-U/246 and hypersensitive reaction to TMV/U1. Therefore these two varieties (Alba Regia and Cecil F1) are very important in pepper breeding and growing *(Table 5)*.

Table 5

Reaction of pepper varieties to different viruses

Varieties	Viruses				
	CMV-U/246	TMV/U1	PVY^{NTN}	SoMV	TSWV
Tuba	+	+	–	–	*
Greygo	+	+	+	–	*
Dabora F1	–	HR	+	–	*
Himes F1	–	HR	+	–	*
Syn. Cecei	–	+	+	–	+
Táltos Synthetic	–	+	+	–	*
Brill F1	+	HR	+	–	*
Fehérözön Synthetic	+	HR	–	–	*
Boni	–	+	–	–	*
Alba Regia	–	+	–	–	–
Gigant F1	–	+	+	–	*
Korona	–	+	–	–	*
Édes alma	–	+	–	–	*
Ciklon F1	–	HR	+	–	*
Tizenegyes	–	+	+	–	*
Cecil F1	–	HR	–	–	*
Rapires F1	+	+	+	–	*
Óriás Cseresznye	–	*	*	*	+
Star	–	+	–	–	*
Keszthelyi Res.	*	HR	*	*	+
Belecskai	*	*	*	*	+
Sobor	*	*	*	*	+
Tüzes Piros	*	*	*	*	–
Kalocsai V2	*	*	*	*	+
Hatvani Erős	*	*	*	*	+
Golden California	*	*	*	*	–

+ susceptible, – resistant, * not investigated, HR hypersensitive reaction

In another experiment 50 commercial Hungarian pepper varieties were tested to four *tobamoviruses* (TMV/U1, ToMV-Ob, ToMV-P8, PMMoV-SL) with different levels of pathogenicity (P_0, $P_{1,2}$, $P_{1,2,3}$). About 20% of the varieties were susceptible to all pathotypes, 45% contained the L^1 gene of resistance, 31% showed high level of resistance (containing L^3 gene) and only one was outstandingly resistant containing L^4 gene (Gáborjányi et al., 1999). Infection of $P_{1,2,3}$ pathotypes means a real threat for the production, because its infection causes only mild symptoms. In the future we have to consider the increase of pathogenicity and the appearance of new, agressive pathotypes both in the fields and the greenhouses. Recently, pepper mild mottle tobamovirus (PMMoV) was also isolated in plastic tunnels (Kálmán et al., 2000). Infected plants did not show visible symptoms but the necrosis of the fruit was frequently observed. The appearance of PMMoV proposes new epidemiological questions, with special regard to pollen and seed transmission of PMMoV (Betti et al., 1986, 1988; Salamon and Kaszta, 2000; Valverde and Landry, 2000).

Sources of resistance

Responses of different pepper varieties, breeding lines, different *Capsicum* species and accessions to virus infections have been extensively studied all the world (Green and Kim, 1994; Mijatovič, 1997; Lane et al., 1997; Castagnoli et al., 1997; Sudarsono et al., 1998; Wang et al., 1998). Out of them new sources of resistance have been found, which could be used for pepper breeding programmes (Sowell, 1982; Lane et al., 1997; Reddick and Habera, 2000).

Intensive research work started in the 1980s in this respect in Hungary. According to our knowledge the resistance of 22 *Capsicum* species to 44 viruses is known (Horváth, 1983; Horváth, 1986a, b, c; Horváth et al., 2000a). Most important incompatible host-virus relations are in *Table 6.*

The main objectives of pepper breeding research are to clear: the resistance genes in *Capsicum* genus; the infectivity of the different virus strains; the inheritance of the resistance genes and crossing possibilities between *Capsicum* species.

Breeding for virus resistance

Resistance breeding is the most effective method against virus diseases. Since the 1980s, the rapid development in the biotechnology and molecular virology gave new possibilities in breeding for virus resistance, in addition to conventional cross-breeding methods (Dudits and Heszky, 2000). Intensive research work has been started all over the world in order to make gene maps of plants and pathogens, to identify, isolate and clone gene(s) responsible for virus resistance and incorporate them into the genome of new varieties. Researchers of Cornell University (Ithaca, USA) have pursued the mapping of the 12 pepper chromosomes. Now they have a detailed genetic map which has allowed them to locate genes and create convenient markers for indirect selection in breeding programs. They use these tools to map and characterize a number of disease resistance genes (Jahn, 2000a). Many papers have been published recently about the genome analysis of pepper (Livingstone et al., 1998, 1999; Paran et al., 1998; Pflieger et al., 1998; Chaim et al., 2001).

Table 6

Resistant and immune *Capsicum* species, hybrids and breeding lines
[after Horváth (1983, 1986a, b, c); Green and Kim (1994)]

Capsicum species	Viruses*
C. angulosum	PVX, PVY
C. annuum	PVY, TEV, TMV
C. annuum 'Criollo de Morelos'	TMV, PVY
C. annuum 'Perennial'	PVY
C. annuum var. longum	CMV
411 *C. annuum* x *C. frutescens*	TMV, PVY
C. baccatum	TMV, ToMV, CMV, PVY
C. baccatum var. pendulum	PVY,TAV,TMV-C/U1, ToMV-Ob
C. cardenasii	PVY
C. chacoense	TMV, ToMV, CMV, PVY
C. chinense	PepMoV, TEV,TMV, ToMV, PVY, CMV, TSWV
C. eximium	PVY
C. flexuosum	PVY
C. frutescens	AMV, CMV, PVY, PVX, TEV, TMV,TRSV, ToMV, TSWV
C. frutescens var. Tabasco	TMV,CMV
413 *C. frutescens* x *C. annuum*	PVY
C. flexuosum	PVY
C. microcarpum	CMV, TMV,ToMV, PVX, PVY
C. pendulum	CMV, PVX, TMV, ToMV
C. praetermissum	ToMV-D/H, TMV, PVX
C. pubescens	CMV, PVY, ToMV, TMV
C. testiculatum	TMV, PVY
255 Pritamin line	TMV, PVY
47/87 F7 R.K. type	TMV, PVY
265 Red chili	TMV, PVY
419 Fűszer USA	TMV
1/96 F2 *C. chinense* x Botond	PVY
420 Fehér alma	PVY
421 F. cseresznye	PVY
424 20/94 DH 6/6b	PVY
425 20/94 DH 6/28	PVY

*CMV, cucumber mosaic virus; AMV, alfalfa mosaic virus; TMV, tobacco mosaic virus; ToMV, tomato mosaic virus; PVY, potato Y virus; PVX, potato X virus; TAV, tomato aspermy virus; TRSV, tobacco ringspot virus; TEV, tobacco etch virus; TSWV, tomato spotted wilt virus; PepMoV, pepper mottle virus

In Hungary, modern breeding based on local varieties began 50 years ago with the activity of Angeli (1918–1971). Among Hungarian pepper breeders Csilléry (1985a, b, c), Csilléry et al. (1983), Moór (1986), Sági and Salamon (1998), Tóbiás et al. (1978, 1982), Zatykó (1982, 1993), Zatykó and Moór (1995, 1998), Márkus et al. (1995, 1998), Andrásfalvy and Csilléry (1995) have done pioneer work in breeding of virus resistance pepper varieties. At the Research Station of the Vegetable Crops Research Institute more

than 50 pepper varieties were bred in the last fifty years. In 1995, 11 varieties contained L^1 and 2 varieties the L^2 genes. Three varieties (Suptol, Táltos, Synth. Cecei) were CMV tolerant and 2 varieties contained L^1 gene and were resistant to CMV infection at the same time (Fehér and Kristóf, 1995). Complex virus infections might cause heavy yield losses depending on the condition of the crop. High importance was attributed to complex resistance in field in reaction to spontaneous virus infections, to yield loss, the extent of which might not coincide with the genetical resistance to certain virus species. Plants resistant in the greenhouse test could react with heavy yield loss to complex virus infections in the field (Márkus et al., 1995).

Breeding for virus resistance started with creating of interspecific hybrids. Different *Capsicum* species served as sources of resistance: L^1 originated from *C. annuum,* L^2 from *C. frutescens,* L^3 from *C. chinense* and L^4 from *C. chacoense.* Possibility of crossing of *Capsicum* species is limited. *C. annuum* as mother plant could be easily hybridized with *C. chacoense,* but it was difficult to cross with *C. chinense* and *C. frutescens.* Reciprocal crossing were more easily done (Zatykó, 1993; Csilléry, 1978, 1983, 1985a).

Intensive breeding work has been started in Hungary in 1968 for TMV resistance. The resistance or susceptibility of wild *Capsic*um species meant the basis to distinguish the pepper pathogen *tobamovirus* pathotypes *(Table 7).* Resistance in *Capsicum* species is ensured by 5, dominantly inherited L gene (L^0, L^1,L^2, L^3, L^4). They are at

Table 7

Relation between pepper genotypes and tobamovirus pathotypes
[after Rast (1979), Boukema (1980, 1984)]

		Pepper tobamovirus pathotypes and viruses*			
		P_0	P_1	$P_{1,2}$	$P_{1,2,3}$
		TMV, ToMV, BePMV, TMGMV, DYFV	ToMV, TMGMV	ToMV, PMMoV	PMMoV
Pepper genotypes					
L^0L^0	S	S	S	S	
L^1L^1	R	S	S	S	
L^2L^2	R	R	S	S	
L^3L^3	R	R	R	S	
L^4L^4	R	R	R	R	

*TMV, tobacco mosaic virus; ToMV, tomato mosaic virus; BePMV, bell pepper mottle virus; DYFV, Dulcamara yellow fleck virus; PMMoV, pepper mild mottle virus; TMGMV, tobacco mild green mosaic virus; S, susceptible; R, resistant

the same locus and not entirely localize the infection. *Tobamoviruses* have four pathotypes (P_0, P_1, $P_{1,2}$, $P_{1,2,3}$). If pepper containing L^0 gene infected with any pathotypes of *tobamovirus*es it resulted compatible host-virus relation. L^1 resistance gene insures resistance against P_0 pathotypes, while L^2 against P_0 and P_1 pathotypes, etc. (Rast, 1979;

Boukema, 1980, 1984). In normal conditions resistance of pepper genotypes to TMV manifested as a hypersensitive reaction (HR), which greatly depended on the influence of temperature, light and some other factors. However a temperature of 30 °C or more might overcome hypersensitive reaction (HR) (Mijatovič et al., 1995; Roggero et al., 1996).

The original source of TMV resistance was "Keyston Resistant Giant", an American variety (Csilléry, 1982). In 1970s the first, Hungarian pepper varieties containing L^1 gene were the following: Novator F1 (pointed hot type), Synthetic Cecei, Fehérözön synthetic, Suptol, Tizenegyes, Rezisztens Keszthelyi (white Cecei types), Greygo and Fecske (tomato shaped). If a pepper variety is grown for a long time containing a resistance gene, the appearance of a new, more agressive, resistance-breaking pathotype of the pathogen could be observed. In 1980s a new pathotype of TMV, so-called Ob strain has been appeared, which has broken the resistance of varieties, containing the L^1 gene (Csilléry and Ruskó, 1980; Tóbiás et al., 1982; Csilléry et al., 1983). The source of resistance in this respect was an interspecific hybrid of *C. annuum* and *C. chinense* (P.I. 159236) (Csilléry, 1985a, b, c). First varieties, containing the L^3 gene were introduced in 1995 (Novares F1, Rapires F1) (Zatykó and Moór, 1995). Recently a new, white pepper variety Ciklon F1 was reported to have the L^3 gene (Sági and Salamon, 1998). To our knowledge the standard variety Bölény from the dark green blocky type was the first in the world to have L^3 gene (Zatykó and Moór, 1995). Linkage between the L^3 gene and an al (anthocyanin less) gene was proved in TMV resistant pepper (Zatykó and Moór, 1998). First hybrid (H 19-6F1) containing the L^4 gene was created by Csilléry in 1993, using *C. chacoense* as a source of resistance which was introduced in 1997 as Hímes F1 (Sági and Salamon, 1998). Since then breeding white, sweet pepper hybrids armed with the *tobamovirus* resistance gene L4 is in progress (Sági and Salamon, 1998). Homozygous L^4 lines having white, sweet fruits of the types 'Cecei', blocky and 'Lamuyo' have been selected in the past years. Using these lines test hybrids were breeded and have been investigated under forced conditions. The allele L^4 was also incorporated into less important blocky and Lamuyo types, having deep green coloured fruits. For preliminary tests each individuals were inoculated with a strain of Dulcamara yellow fleck *tobamovirus* (DYFV) (syn: Ob strain of tomato mosaic *tobamovirus*). Resistant plants were screened in excized leaf tests for the presence of the allele L^4 using a strain of PMMoV that breaks the resistance allele L^3. In many countries we have seen that resistance based on the alleles L^1 and L^3 has been broken. For this reason many breeding companies are trying to breed for PMMoV (pathotypes 1, 2, 3) resistant varieties, by using the L^4 allele (Van Duin, 1998).

CMV resistance inherits by polygenic recessive way, while PVY resistance by monogenic recessive way, therefore resistance breeding against these viruses is more difficult, as compared with breeding against *tobamoviruses*. *C. frutescens* 'BG2814-6' and *C. annuum* 'Perennial', the leading source of CMV tolerance, appear to share one or more CMV resistance genes (Grube et al., 1996). CMV was detected in non-inoculated leaves in a small percentage of both BG2814-6 and 'Perennial plants', indicating that resistance is not absolute in either genotype. Serological tests suggest that there may be a mechanistic difference in resistance between the two genotypes (Grube et al., 2000a). Besides these, *C. baccatum* var. pendulum PI 1 15421 as a new source of moderate field resistance to CMV

for pepper breeding should be taken into account in future research (Castagnoli et al., 1997). In addition to these, tools were developed for marker-assisted selection (MAS), to facilitate further transfer of CMV resistance (Grube et al., 1996, 2000b).

Breeding for *potyvirus* resistance has not yet considerable results in Hungary. Among *potyviruses* only three [tobacco etch *potyvirus* (TEV), pepper mottle *potyvirus* (PepMoV) and PVY] are dominant in Europe and North America (Caranta et al., 1998; Grube et al., 1998, 2000a, b). Among them only PVY has economic importance in Hungary. Several resistance genes for *potyviruses* are known in *Capsicum* genus. Caranta et al. (1995) used doubled-haploid lines and molecular markers for genetic dissection of the complex resistance of *C. annuum* 'Perennial'. The multipotyvirus resistance from 'Perennial' was shown to result from the association of several additive and epistatic genetic factors with partial (quantitative) effects. Some of these genetic factors were virus- or strain-specific, others were more polyvalent and confer partial resistance to different viruses. Some partial resistance factors cosegregated with major genes for absolute resistance to chilli veinal mottle *potyvirus* (CVMV) and PVY. This allowed to establish a structural as well as functional continuum between monogenic, oligogenic and polygenic resistance systems. Caranta et al. (1997) reported the molecular mapping of *potyvirus* resistance factors from pepper line 'Perennial' and an analysis of the genetic basis of multipotyvirus quantitative resistance, which allows us to make comparisons with the location of major genes and quantitative trait loci (QTLs) involved in virus resistance. Dogimont et al. (1996) studied the genetic basis of the broad spectrum resistance to *potyvirus*es in a Mexican pepper line (CM 334) using doubled-haploid lines. Segregation analyses indicated that resistance to pepper *potyvirus*es in CM 334 was conferred by two genes. The first one, tentatively named *Pr4,* was dominant and confered the resistance to all known pathotypes [P(0), P(1), P(1,2)] of PVY and to PepMoV, although, other gene(s) were also present, conferring resistance at different level (Andrés et al., 1998). The second one, named Pr5, was recessive; it confered only the resistance to common strain [P(0)] of PVY. Kyle and Palloix (1997) recently proposed the revision of the nomenclature for genetic loci in *Capsicum,* governing *potyvirus* resistance. At present there is clear evidence for four independent loci in *Capsicum,* each with alleles that confer resistance to viral isolates that belong to one or more of the viruses: PVY, PepMoV and TEV. Pasko et al. (1995) reported a cytoplasmically determined resistance to PVY in *C. annuum* 'Puerto Rico Wonder'. Other studies determined the mechanism (Deom et al., 1997) and inheritence (Berke et al., 1998) of *potyvirus* resistance, e.g. the resistance of *C. annuum* cv. Dempsey to TEV was due to interference with virus RNA accumulation (Deom et al., 1997).

Resistance breeding against TSWV resulted hypersensitive response of some *Capsicum* varieties with resistance introgressed from different accessions of *C. chinense* (Black et al., 1991; Boiteux, 1995; Hobbs et al., 1994; Nuez et al., 1994). Progeny testing and allelism tests allowed to assume that the resistance of more accessions is monogenic, dominant and located at the same locus named *Tsw* (Boiteux, 1995). Resistance in the resistant accessions is caused by a restriction of cell to cell movement and depends on phenological stage. When plants were inoculated at an early vegetative stage, the virus could reach the vascular system causing systemic infection. When plants were inoculated

at a 8–12 leaves stage, the infection was more confined and prevented systemic infection (Soler et al., 1998, 1999). Jahn et al. (2000b) has made genetic mapping of the Tsw locus for resistance to TSWV in *Capsicum* genus. Some isolates of TSWV and continuous high temperature might overcome the hypersensitive response of *Capsicum* varieties (Moury et al., 1995; Roggero et al., 1996, 1999). The experiments reported are of practical interest because cultivated species of *Capsicum* are susceptible to TSWV and are generally grown in plastic tunnels where temperatures often exceed 30 °C. Low temperature could also break resistance of *C. chinense* to TSWV (Arteaga and Ortega, 1998).

Regarding that viruses always occur in complex infection, breeding pepper cultivars for multivirus resistance is of great importance (Cook et al., 1976; Hamza et al., 1998).

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Flower Colour Breaking in Two *Aubrieta* Cultivars Caused by Cucumber Mosaic *Cucumovirus* (CMV) Lacking Satellite RNA-5

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Cucumber mosaic *cucumovirus* (CMV), isolate Aub (CMV-Aub), was the only virus found in plants of *Aubrieta* cvs Blue Emperor and Gloriosa showing symptom of flower colour breaking. CMV-characteristic electrophoretic banding pattern of replicative RNA (dsRNA), lacking satellite RNA-5 (CARNA5), was easily recognized, especially when the RNA was purified from experimentally infected *Nicotiana glutinosa* L. and *N. megalosiphon* Heurck et Muell. plants, but also from original hosts. The pattern was absent in symptom-free *Aubrieta* plants. Virus identification was supported, in addition, by test plant and serological reactions. CMV-Aub is a rather unstable isolate; its particles were detected in purified preparations before and after density gradient centrifugation, however, only in fixed preparations (1% formaldehyde). The virus induced mild leaf symptoms in *Cucumis sativus* L. cv. Delicatess. No difference was found between virus isolates from the two *Aubrieta* cultivars. There were also indications of virus presence in *Aubrieta* cvs Gartenstadt Purpur and Vesuv.

Keywords: *Aubrieta* cultivars, cucumber mosaic *cucumovirus*, lack of RNA-5.

Worldwide, cucumber mosaic *cucumovirus* (CMV) has been found spontaneously to infect several hundred wild and cultivated species belonging to more than 60 plant families (Horváth, 1979, 1980, 1993b; Klinkowski, 1977a, b; Kaper and Waterworth, 1981). The virus has an extremely broad host range, infecting 85 distinct plant families, and up to 1000 species experimentally (Van Regenmortel et al., 2000). Many isolates were demonstrated regarding some specific host range and symptom differences. Two serological groups of strains are known, as well as differences among numerous virus isolates with regard to virus satellite RNA population composition, particularly with respect to the presence of RNA-5 (Francki et al., 1979, 1987; Kaper and Waterworth, 1981; Bos, 1999). Some isolates of CMV on one hand and of tomato aspermy (TAV) and peanut stunt *cucumoviruses* (PSV) on the other are somewhat similar in antigenic (in general, at a low degree) and some symptom properties (Kaper and Waterworth, 1981; Brunt et al., 1996; Van Regenmortel et al., 2000).

CMV provokes flower colour-breaking symptom in a series of plants, including *Chrysanthemum, Matthiola, Petunia, Primula, Vinca, Zinnia, Viola, Gladiolus, Ranunculus* and *Campanula* species, possibly a *Crocus* species (Smith, 1972; Klinkowski, 1977b; Valverde, 1984; Horváth, 1993a, b; Loebenstein et al., 1995; Bos, 1999) and some other species.

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In Croatia CMV infections have been recorded in many wild, vegetable and ornamental plants (Šarić and Štefanac, 1988; Juretić, 1994). Sometime we isolated CMV in this region from two *Aubrieta* cultivars that exhibited flower colour breaking. Those cultivars and the cultivars Gartenstadt Purpur and Vesuv (see Material and Methods and Discussion) probably belonged to the species *Aubrieta deltoidea* (L.) DC., however, hybrid plants, e.g. *A. hybrida* Haussknecht (a hybrid between *A. deltoidea* and *A. gracilis* Spruner et Boiss.), or another hybrid, between *A. deltoidea* and *A. erubescens* Griseb., also could be taken into consideration here (Beckett, 1984; Cullen et al., 1995; Tutin et al., 1996). In addition to experiments with test plants and serological experiments within the virus identification procedure, the present paper deals with the analysis of virus RNA profile and stability properties of virus particles.

Aubrieta species (aubrieta, purple rock-cress), family Brassicaceae (formerly: *Aubrieta*, family Cruciferae) are evergreen trailing or mat-forming perennials native to the mountainous region from Italy to Iran; as horticultural plants they are grown much more widely (Beckett, 1984; Cullen et al., 1995; Tutin et al., 1996). Several viruses have been found to infect about 12 of the species spontaneously, CMV and tomato black ring *nepovirus* (TBRV) being the most common (Klinkowski, 1977a, b, c).

Materials and Methods

Within a few comparatively small populations of *Aubrieta* cvs Blue Emperor and Gloriosa plants cultivated in the open some of them potted in the Botanical Garden of the Faculty of Science, University of Zagreb – the seeds having been obtained from the Botanical Garden of the University of Vienna in early 1990s – a small percentage (exceptionally up to 15%) of both cultivars displayed flower colour breaking (observed during April and May and sometimes also during the end of March). A few areas of more or less sharply separated shades of violet or purple, lighter and darker than healthy flower colour – those darker often extending along the petal main veins – were present on some or all of the petals of individual flowers *(Fig. 1)*. Slight petal malformation was present sporadically. Sometimes areas of mild chlorosis and also weak malformation and stunting were present on middle-aged and younger leaves. From such specimens inocula were prepared separately from both cultivars for mechanical inoculation of the test plants *Chenopodium quinoa* Willd., *Nicotiana glutinosa* and *N. megalosiphon*. Leaves of *Aubrieta* plants were triturated without or with the addition of several petals, either in 50 mM pH 7.5 Sörensen's phosphate buffer containing 1 mM NaEDTA or 0.1% thioglycolic acid (TGA), or in tap water alone, at a ratio of ca. 1:15 (w/v). Such a dilution of the inocula was necessary because of a high content of mucilaginous substances in the leaves. Virus isolates were maintained in a glasshouse in the mentioned *Nicotiana* spp., in daylight and at temperatures between 20 and 35 °C. When preparing inocula from leaves of the latter plants using either the buffer or more often water, a ratio of 1:1–2 (w/v) was employed.

Analysis of virus replicative RNA followed the procedure of Valverde et al. (1990) including modifications introduced by Krajačić and Lorković (1992). Specimens



Fig. 1. Colour-breaking symptom in a flower of an *Aubrieta* 'Blue Emperor' plant spontaneously infected with isolate CMV-Aub of cucumber mosaic *cucumovirus* (CMV)

of the two spontaneously infected *Aubrieta* cultivars as well as those of artificially infected *N. glutinosa, N. megalosiphon* and *Cucumis sativus* cv. Delicatess were separately processed. Five-gram portions of frozen leaf tissue were ground in 8 ml of STE extraction buffer (0.1 M NaCl, 50 mM tris, 1 mM NaEDTA), pH 6.8. After low speed centrifugation of the homogenate the supernatant was stirred vigorously for 30 min with 2 ml of 2-mercaptoethanol, 1 ml of 10% SDS, 3 ml of neutralized water-saturated phenol and 2 ml of chloroform. Following centrifugation of the emulsion at 4,000g for 15 min the water phase was collected and adjusted to 16% of ethanol (EtOH) content.

From total nucleic acid extracts dsRNA was further purified by chromatography. The samples were poured onto columns of 16% EtOH-equilibrated cellulose CF-11 (0.5 g of dry weight per column). After washing with 40 ml of STE + 16% EtOH dsRNA was released and collected with 6 ml of EtOH-free STE. Precipitation of purified dsRNA was carried out by adding 0.5 ml of 3 M sodium acetate pH 5.5 and 20 ml of 95% EtOH to each sample and storing overnight at –20 °C. dsRNA was pelleted by low speed centrifugation and resuspended in 20 µl of electrophoretic sample buffer (10 mM tris, 1 mM NaEDTA, 0.25% SDS, 0.03% bromphenol blue, 0.03% orange-G, 15% RNAse-free sucrose, pH 7.5).

Electrophoresis was running at a constant potential of 100 V for an hour in a horizontal mini slab-gel of 1.2% agarose in TBE buffer (90 mM tris, 90 mM boric acid, 1

mM NaEDTA; pH 8.3). Gel was stained for 10 min in 50 ng/ml of ethidium bromide and visualized on a UV transilluminator at 300 nm wavelength. The reference isolate in these and other experiments was a CMV-Cr isolate from tomato plants with lethal necrosis syndrome (Škoric et al., 1996).

Infected *N. glutinosa* plants were the source for purification of both *Aubrieta* virus isolates. Systemically infected leaves (10 g) were homogenized in an equal volume of 67 mM, pH 7.3 Sörensen's phosphate buffer containing 1 mM NaEDTA and 0.1% TGA. The homogenate was emulsified for 10 min with 5 ml of chloroform, the mixture centrifuged at 4,000g for 10 min and then the supernatant for 90 min at 90,000g. The pellets were resuspended in a small volume of 20 mM phosphate buffer pH 7 and the suspension was centrifuged at a low speed. A portion of the supernatant fluid, i.e. partially purified virus suspension, was fixed by adding to it dropwise an equal volume of 2% formaldehyde.

For density gradient centrifugation, continuous RNAse-free 10–40% sucrose gradient was prepared in the virus-resuspending buffer. Running took four hours at 24,000 rpm in a 'Beckman' SW 25.1 rotor. Gradients were analysed at 254 nm wavelength in an ISCO, model 640, density gradient fractionator and UA-5 absorbance monitor.

Preparations for electron microscopy were stained in 1% phosphotungstate at pH slightly above 7.0 and examined in an Opton I electron microscope.

Serological double-radial immunodiffusion experiments were conducted in 1% agarose gel in distilled water. Phosphate buffer, 20 mM pH 7.3 containing 0.1% TGA, was utilized in preparing plant *(N. megalosiphon)* sap of infected or healthy leaves. Test samples examined included: low speed centrifuged leaf sap of plants systemically infected separately with virus isolates from both *Aubrieta* cultivars and the carnation isolate of CMV (CMV-Cr) as reference isolate, of healthy plants and also unfixed and fixed partially purified *Aubrieta* isolates. Rabbit polyclonal antiserum (titre 1/64) to a CMV-Cr prepared earlier by Dr. E. Luisoni (Istituto di Fitovirologia Applicata, Torino) was used; dilutions of it were prepared in the 67 mM, pH 7.3 buffer without any ingredient.

Results

Test plant reaction

The infectivities of inocula prepared from Blue Emperor and Gloriosa cultivars in both phosphate buffer and in water were comparable and symptoms in test plants were indistinguishable.

In *C. amaranticolor* Coste et Reyn. and *C. quinoa* symptoms developed only in inoculated leaves: chlorotic lesions with a minute necrotic centre and chlorotic, ochre or necrotic lesions, respectively. Lesions in inoculated leaves, necrotic in *N. glutinosa* and chlorotic, often with a necrotic margin, in *N. megalosiphon,* were accompanied by systemic mosaic and leaf malformation. Sporadic systemic more or less diffuse chlorotic rings developed in *Datura stramonium* L. In *C. sativus* chlorotic lesions arose in inoculated leaves as did vein clearing, vein banding and mosaic of mild to moderate intensity in younger ones.

dsRNA analysis

Electrophoretic profiles of dsRNA preparations from naturally infected 'Blue Emperor' and 'Gloriosa' plants contained several nucleic acid bands *(Fig. 2)*. A characteristic banding pattern of the CMV-dsRNA was relatively easily recognized by comparing four more prominent bands to those of CMV-Cr isolate. Some additional dsRNA bands, probably of host plant origin, were situated mainly between virus dsRNA-3 and dsRNA-4 bands. *Aubrieta* plants with flowers of normal appearance yielded no virus electrophoretic bands. As expected, transfer of the isolates to *N. glutinosa* and *N. megalosiphon* significantly intensified CMV-dsRNA bands; contrarily, the intensity of the additional bands mentioned was considerably reduced *(Fig. 2)*. In preparations of both *Aubrieta* and *Nicotiana* plants no trace of satellite dsRNA-5 was detected, in contrast to a very prominent band in the CMV-Cr isolate.

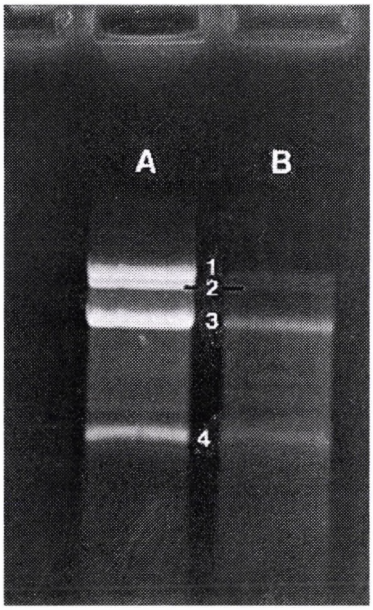


Fig. 2. Agarose gel electrophoresis of dsRNAs from plants infected with isolate CMV-Aub: lane A = experimentally infected *Nicotiana megalosiphon;* lane B = naturally infected *Aubrieta* cv. Blue Emperor; 1, 2, 3, 4 = virus dsRNA-1, dsRNA-2, dsRNA-3, dsRNA-4; Magnification: x 1.6

Some differences concerning the intensity ratio among particular virus dsRNA bands from different hosts were observed. Both naturally infected aubrietas and experimentally infected *Nicotiana* spp. revealed dsRNA-3 band intensity as the highest. In aubrietas the intensity of dsRNA-1 and dsRNA-2 was exceeded by that of dsRNA-4, whereas the opposite was true in *Nicotiana* plants *(Fig. 2)*. It was not possible to recover the characteristic CMV-dsRNAs from younger leaves of *C. sativus* that nevertheless showed clear symptoms of infection.

Properties of virus particles

Virus particles could be detected by electron microscope only in fixed partially purified preparations. It was similar with the detection of virus sedimenting zones in density gradients. In addition to the upper zone located just below the gradient meniscus, only the mentioned preparations revealed a lower (virus particle) zone which, however, seemed to be heterogeneous, as followed from the shape of UV absorption peak.

Serological experiments

Weak and somewhat diffuse precipitation lines were obtained with both clarified and purified (in the latter ones only those formaldehyde-fixed) *Aubrieta* isolates. They were slightly more intense with purified preparations, but still weaker than those with the CMV-Cr isolate. Low intensity precipitation of normal plant proteins was obtained with clarified healthy and infectious sap, and undiluted or 1/2 diluted antiserum.

Discussion

Several *Aubrieta* species, among them *A. deltoidea* and *A. deltoidea* var. graeca (Griseb.) Regel, infected with CMV and possibly all of them found as showing the symptom of breaking of flower colour have been recorded (e.g. Blattný and Schmelzer, 1976; Fletcher, 1987; reviewed by Klinkowski, 1977a). In brassicaceous plants such virus-induced symptom is known to be caused mainly by turnip mosaic *potyvirus* (Tomlinson, 1970; Klinkowski, 1977b; Cooper, 1993; Loebenstein et al., 1995). At least 30–40 other viruses are known to induce in various plants the symptom (flower colour-break, flower-breaking or breaking of petal colour), i.e. mosaic or variegation of flower petals (Bos, 1970, 1999; Smith, 1972; Horváth et al., 1975; Klinkowski, 1977a, b, c; Cooper, 1993; Horváth, 1993a; Loebenstein et al., 1995; Chen et al. 2000, etc.).

After virus replicative RNA analysis placed our two isolates from 'Blue Emperor' and 'Gloriosa' plants in the *Cucumovirus* genus, serological reactions together with test plant response (cf. Gibbs and Harrison, 1970; Kaper and Waterworth, 1981) – especially *C. sativus, D. stramonium, Nicotiana* spp. – confirmed their CMV identity. No significant difference was found between our two isolates, so for both we will use a common designation: isolate CMV-Aub (simply: Aub). Recently, we detected flower colour breaking in several specimens of *Aubrieta* cvs Gartenstadt Purpur and Vesuv growing within a comparatively bigger and a small, respectively, population in the mentioned Botanical Garden in Zagreb. The seeds of the plants were obtained as described in Materials and Methods ('Gartenstadt Purpur'), and that of 'Vesuv' originated from a Vienna park, Wien-Belvedere (in 1996). Preliminary investigations indicated that a symptom, of similar appearance to that in cvs Blue Emperor and Gloriosa, was also caused by CMV without associated RNA-5. We do not know whether the virus (CMV) was contained in the obtained *Aubrieta* seeds, or the plants were infected in Zagreb with some CMV local isolate.

As in our previous work (Krajačić and Juretić, 1993; Škorić et al., 1996; Hrženjak et al., 1999), the present results proved virus replicative RNA analysis to be a powerful

tool for detection and starting identification of CMV. A lower dsRNA concentration, as expected, in naturally infected *Aubrieta* plants was sufficient for virus detection dealing with an increased but still practical amount of tissue. The production of virus dsRNA reached in case of CMV-infected *Nicotiana* plants an extremely high level. As could be estimated from the electrophorogram, a starting amount of 1 g or even less of infected tissue would yield a recognizable dsRNA pattern.

Differences in the intensity ratio of virus dsRNA bands from *Aubrieta* and *Nicotiana* plants revealed what was probably a different strategy in virus genome expression in different hosts. The relative proportions of the encapsidated RNA components have been found to vary considerably with virus strain, host plant and several other conditions (Kaper and Waterworth, 1981). How these factors influence the RNA-component ratio in CMV particles is not completely understood. The amount of replicated nucleic acid species available – represented by quantity of double-stranded form – may be the only or the main factor governing the amount of encapsidated RNA.

According to Kaper and Waterworth (1981), 'the procedures used to purify various strains of *cucumoviruses* are nearly as varied as the strains involved', factors that affect CMV yield having been reviewed by Yarwood (1971). In our attempt we combined some steps and conditions from protocols which proved satisfactory for most CMV strains (Francki et al., 1979). The absence of a virus sedimenting zone in unfixed preparations after density gradient centrifugation suggested that we were dealing with a rather unstable isolate. The heterogeneous virus zone obtained with fixed preparations could be due to particle damage and aggregation. This and the abundant material included in surface zone close to meniscus, which originated probably not only from the host protein but also from constituents of disrupted virus particles, speaks in favour of the isolate Aub particle instability. The low intensity and especially the appearance of isolate Aub serological reactions could be taken, at least partly, as a result and also indicator of that instability.

Acknowledgement

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Zucchini Yellow Mosaic Virus and its Vectors in Cucumber in Western Hungary

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Survey was carried out in three supported cucumber fields to monitor the spread of *Zucchini yellow mosaic virus* (ZYMV). Aphid flight was monitored by yellow water pans. ZYMV vector aphid species were present in equally high numbers in different sites. ZYMV infestation occurred only in one field. Plants showing symptoms of the virus were regularly destroyed. In spite of the regular eradication of the infection sources, new plants became infected during the whole season. ZYMV infected plants as initial internal virus sources played prominent role in starting the epidemiology of the virus in the field.

Keywords: *Acyrtosiphon pisum, Aphis craccivora, Aphis gossypii,* cucumber, *Myzus persicae, Zucchini yellow mosaic virus.*

In Europe *Zucchini yellow mosaic potyvirus* (ZYMV) was first described from Italy by Lisa et al. in 1981. It has been recorded from France, Germany, Israel, Lebanon, Morocco and Spain (Lisa and Lecoq, 1984), England, Turkey, Jordan, Egypt, Japan, Taiwan, Australia, Canada and from the United States (Sammons et al., 1989). ZYMV was recorded in the Netherlands, but by strict elimination of infected plants the virus was successfully eradicated (Schrijnwerkers et al., 1991). ZYMV causes very serious economic damage in California (Perring et al., 1992; Grafton-Cardwell et al., 1996).

In Hungary *cucumber mosaic cucumo virus* (CMV) was the prevailing virus disease of cucumbers (Basky and Nagy, 1980; Basky 1984a). Watermelon mosaic *potyvirus* (WMV) was also present but it occurred less often (Basky, 1982, 1983a). Even if the proportion of virus infected plants reached 50% by the end of the vegetation period economic damage caused by CMV and WMV was negligible (Basky, 1983a, 1983b). *Zucchini yellow mosaic potyvirus* was first detected in Hungary by Tóbiás et al. (1996). Basky and Tóbiás (1998) studied the spread of ZYMV within a zucchini field. ZYMV often occurred in mixed infection with CMV and WMV in their study. Here we report the results of surveys carried out in western Hungary.

Materials and Methods

Survey was carried out in the most important pickled cucumber growing area in Western Hungary in Csorna, Sopronnémeti and Szárföld. The cucumbers were transplanted in 3 leaf stage in the middle of May, the fields were irrigated, and kept weed free during the

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vegetation period. The following cultivars were grown in different locations as supported cucumbers: Csorna: Accordia and Harmonie, Sopronnémeti NU-81-05, Szárföld: Duett and Harmonie. Fields were surveyed from 28 May twice weekly till 4 August. The number of colonizing aphids were counted on 100 leaves, and number of plants showing symptoms typical for ZYMV infestation (malformation, yellow flecks, blistering, leaf narrowing, stunting) were recorded. Leaves of symptom showing plants were collected for virus identification by DAS ELISA (Clark and Adams, 1997).

Moericke yellow water pans were placed in the middle of each field at canopy level, pans were emptied twice a week, aphids were stored in 70% ethanol until identification. Aphids were identified under stereo microscope.

Results and Discussion

Colonizing aphids

The cucumber aphid (*Aphis gossypii* Glov.) formed small colonies on cucumbers in our experimental fields. The first aphid colonies occurred on 25 June each site *(Fig. 1),* but the size of colonies was small. Most cases the colonies consisted in one aptera and its progenies. It is in agreement with the results of Basky (1984a) and Nasser and Basky (1988): under field conditions they never found large aphid colonies in cucumbers. According to our study small colonies were present till the end of the season.

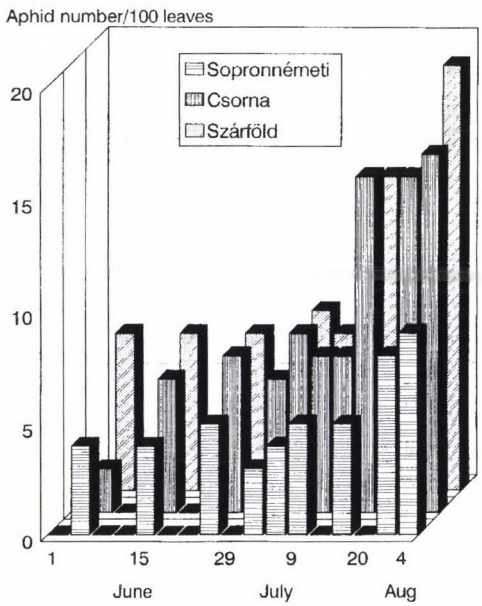


Fig. 1. Number of aphids on 100 leaves

Virus infection

The field survey revealed the presence of *zucchini yellow mosaic virus* in one of our experimental fields in Szárföld. The first symptom showing plants were recorded on 11 June, DAS ELISA revealed the presence of *zucchini yellow mosaic virus* in the leaf samples collected from symptom showing plants. To decrease the damage caused by ZYMV; the infected plants were eradicated. In spite of the severe phyto-sanitary control newly infected plants occurred in slightly increasing number at each survey till the end of the season *(Fig. 2)*. The occurrence of newly infected plants is due to the virus transmission of aphids from internal sources. It happened, because the development of characteristic leaf distortion, malformation and blistering requires one month (Basky and Tóbiás, 1998). Symptom showing plants were destroyed, but they served as internal virus source before characteristic ZYMV symptoms developed.

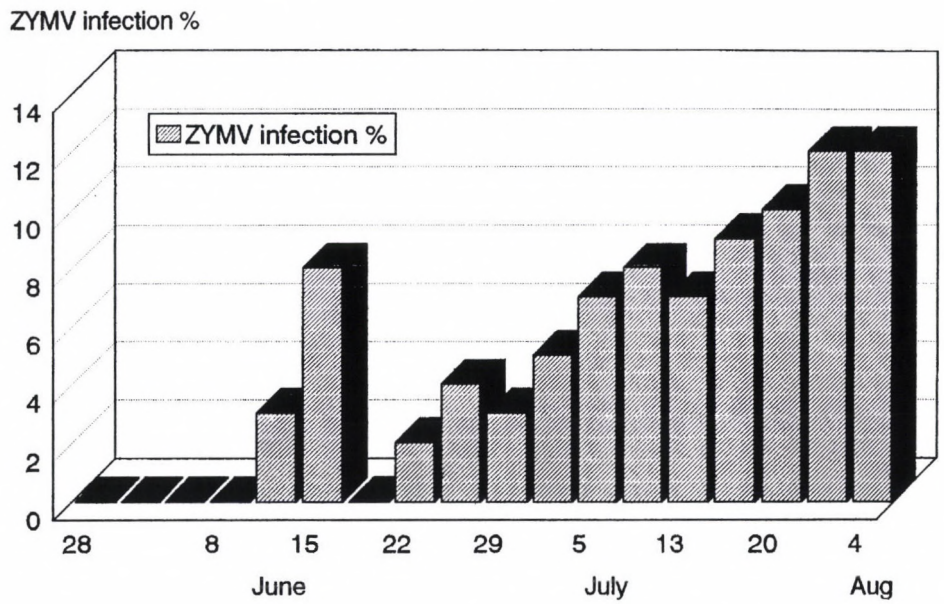


Fig. 2. Percentage of ZYMV infected plants (Szárföld, 1999)

Alate aphid flight activity

Aphis gossypii Glover efficiently transmitted ZYMV (Lisa et al., 1981). Adlerz et al. (1987) found that *Myzus persicae* Sulzer, *Acyrthosiphon pisum* (Harris) and *Aphis craccivora* Koch were efficient vectors of ZYMV. Apart from *Aphis craccivora* the most important ZYMV vector aphid species were present in all locations. The most important ZYMV vector aphid species were very abundant in the yellow pans *(Figs 3, 4, 5)*. In spite the intensive vector flight ZYMV infestation occurred only in Szárföld, there was no sign of ZYMV infestation in the cucumber fields in Csorna and Sopronnémeti. The lack of

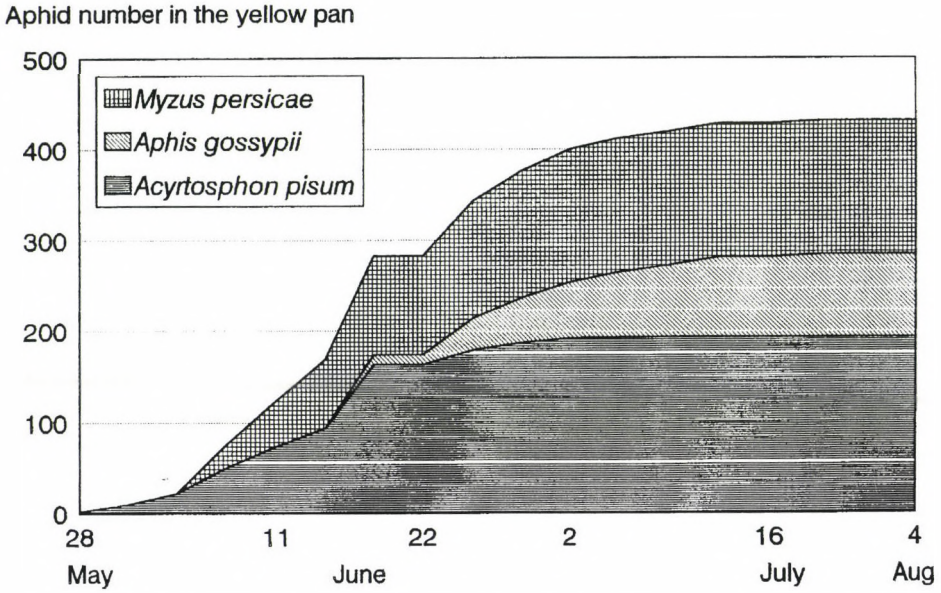


Fig. 3. Cumulative number of ZYMV vector aphid species in the yellow pan (Szárföld, 1999)

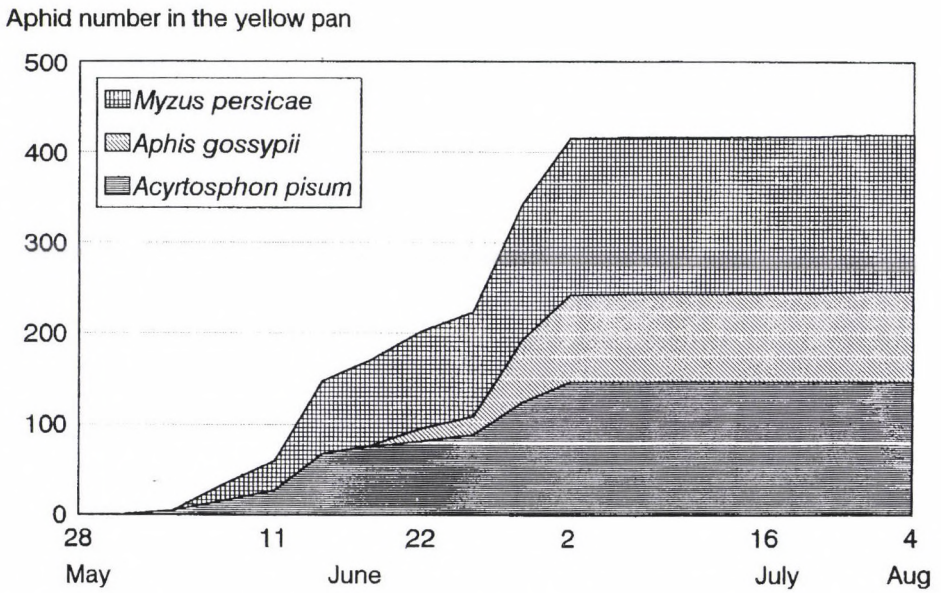


Fig. 4. Cumulative number of ZYMV vector aphid species in the yellow pan (Sopronnémeti, 1999)

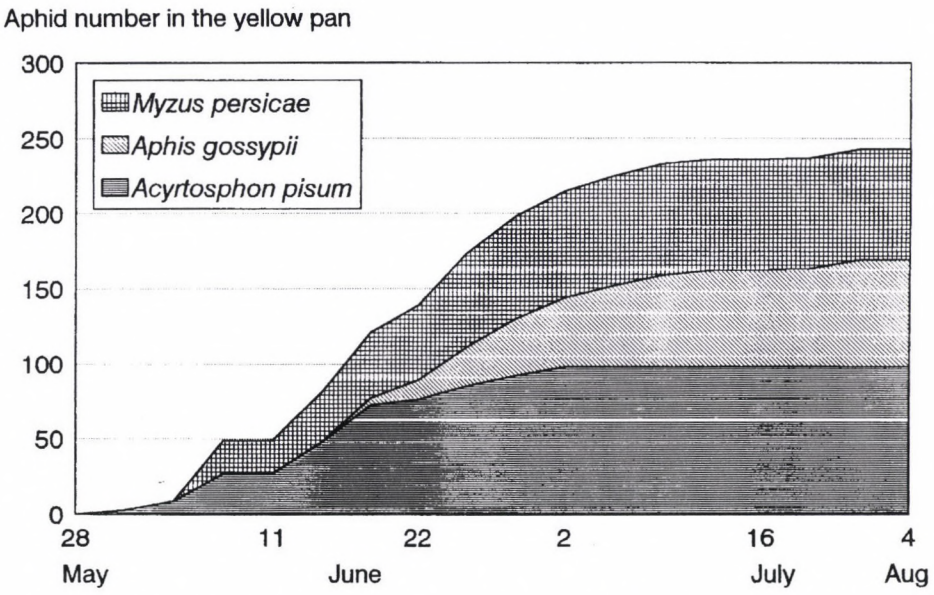


Fig. 5. Cumulative number of ZYMV vector aphid species in the yellow pan (Csorna, 1999)

ZYMV infestation in Csorna and Sopronnémeti is due to the absence of the initial virus source. Apart from aphid transmission, ZYMV can be transmitted in a very low proportion by seeds. Most probably the whole ZYMV epidemy started from a single infected plant in Szárföld, which was the result of a seed transmission. The single infected plant served as initial virus source for further aphid transmission. Although the symptom showing plants were regularly destroyed; new infections occurred during the whole season, because the characteristic blistering, leaf narrowing and mottling appears on the infected plants 3–4 weeks after infestation. Plants showing mild mosaic symptoms 7–10 days after infestation were already suitable sources for vector species to acquire the virus. It has been proved by Basky and Tóbiás (1998) they found close correlation between number of ZYMV infected plants and cumulative number of ZYMV vector aphid species one month earlier. There was no correlation between the proportion of (CMV and WMV) infected plants and number of aphids when the time of symptom development was not considered (Basky, 1986; 1993). In our experiment the plants showing characteristic ZYMV symptoms were removed from the field therefore it was impossible to calculate the relationship between number of vector aphids and number of ZYMV infected plants. However the importance of initial internal virus source in development an epidemy is proved by our study, the aphid flight activity was about the same in the three sites, ZYMV infection occurred only in one place where the initial internal virus source was present. The infection could not come from reservoirs, because ZYMV is not widespread in Hungary yet. Under our continental climate *Cucurbitaceae* hosts of ZYMV are not able to survive the severe winters unlike in California (Perring et al., 1989) therefore in Hungary plants emerging from infected seeds have major importance in initiating the epidemiology of ZYMV.

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Molecular Polymorphism between Population of *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) Propagating on Tobacco and Onion

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The molecular polymorphism between the specimens of *Thrips tabaci* Lindeman collected from tobacco *(Nicotiana tabacum)* and from onion *(Allium cepa)* was observed by RAPD-PCR-analysis. The amplified DNA-banding patterns of *T. tabaci* populations propagate on tobacco were significantly different from those propagate on onion. In total, 54 amplified DNA-bands were scored. Of these, 39 (72.0%) were different, and 7 (13.0%) bands were mutual between the two populations. A slight molecular dissimilarity between females and larvae of *T. tabaci* populations propagating on tobacco and onion were observed on the basis of one (1.9%) discriminating PCR-fragment and six ones (11.1%), respectively. The results presented indicate an intraspecific molecular variability between populations of *T. tabaci* propagating on tobacco as well as on onion.

Keywords: *Thrips tabaci communis, Thrips tabaci tabaci,* PCR., tobacco, onion.

According to the statement of Zawirska (1976) *Thrips tabaci* Lindeman comprises two "types", from among which the populations of *T. tabaci tabaci* living on *Nicotiana tabacum,* and out of the vegetation period on different weed species, propagate by arrhenotoky and are able to transmit tomato spotted wilt tospovirus. The populations of *T. tabaci communis* living on different plant species, mainly on onion, they propagate with teletoky and their specimens are not virus vectors. Schliephake and Klimt (1979) use the subspecies expression. Since Klein and Gafni, (1996) as well as Kraus and coworkers (1998) had found intraspecific molecular differences between Thysanoptera species as well as Thysanoptera populations occurring in different agrobiotopes, we have begun to investigate whether there are possible intraspecific molecular differences between the two *T. tabaci* types (subspecies), in spite of morphological similarity of adults.

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Materials and Methods

Sampling of thrips

Thrips individuals were collected from two populations from tobacco and onion. Specimens living on tobacco (70 females and larvae), were collected at Kállósemjén, (Hungary), July 13 2000, comprising about a fifty sampling sites across the 10 ha tobacco plantation. Specimens living on onion (70 females and larvae) were collected at Tordas (Hungary), July 19 2000 across a 1/2 ha plantation comprising about twenty sampling sites. Sexes, and developmental stages were selected under dissecting microscope. Samples were stored in DNA extraction buffer at –20 °C until DNA extraction.

DNA extraction

DNAs of pooled samples of females and larvae of both populations were extracted according to the CTAB-protocol of Doyle and Doyle (1990) with the modification of Mendel et al. (1994). Thrips were ground to fine powder under liquid nitrogen prior to adding 400 µl of DNA extraction buffer followed by an incubation in water bath for 30 min at 60 °C. An equal volume of 400 µl chloroform: isoamyl alcohol (24:1) were added to each sample at room temperature followed by a separation in microcentrifuge at 12000 r.p.m., 4 °C, for 3 min. DNA samples were precipitated from the aqueous phase by an addition of a 2/3 volume of isopropanol, washed by 70% ethanol, let them dry for 30 min. at room temperature, and resuspended in 100 µl $T_{10}E_1$ buffer (10 mM Tris, 1 mM EDTA). The quality of isolated DNA was determined on a 0.8% agarose (SIGMA) gel stained with ethidium bromide (0.5 ng/µl).

RAPD-PCR analysis

For PCR analysis thirteen decamer primers (Operon Tech) were used: OPA/01 (CAGGCCCTTC), OPA/08 (GTGACGTAGG), OPA/09 (GGGTAACGCC), OPA/11 (CAATCGCCGT), OPA/16 (AGCCAGCGAA), OPA/19 (CAAACGTCGG), OPH/11 (CTTCCGCAGT), No/11 (ACGGCATATG), OPAB/09 (GGGCGACTAC), OPAI/21 (CACGCGAACC), OPAL/20 (AGGAGTCGGA), PAL/1 (GCGATGTAGCG), PAL/2 (CCAGGTGGACC). All of these primers were taken from a pre-tested primer collection which produced extremely reproducible PCR band patterns in a previous study. PCR amplification was carried out in a 25 µl reaction mixture containing 10–20 ng genomic DNA, 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 0.75 µM d NTPs, 60 nM primer, 1.5 U Taq DNA polymerase (Gibco). Cycling was carried out in a Perkin-Elmer 9600 thermocycler with the following steps: 1 cycle of 94 °C for 2 min.; 40 cycles of the steps of 94 °C for 10 sec, 36 °C for 30 sec, 72 °C for 1 min.; and closed by a cycle of 72 °C for 2 min. Electrophoresis was performed in 1.6% agarose (Gibco) gels. PCR fragments at low intensities were only scored as present when they were reproducible in repeated experiments. A negative control which contained all the necessary PCR components except template DNA was also included in PCR runs. Each successful reaction with scorable bands was repeated at least twice. Fragment sizes were estimated by comparison to DNA molecular weight markers (Boehringer Mannheim, and Fermentas).

Results

All of the thirteen RAPD primers applied resulted in a highly scorable PCR fragment patterns. The number of amplified DNA bands varied from 1 (primer OPA-11, *Fig. 1*) to 9 (primer No/11, *Fig. 1*) per primer with molecular weights from about five hundred to 2000 base pairs. In total, 54 (100%) amplified DNA bands were scored. Of these, 39 (72.0%) were found to be significantly different, and 7 (13.0%) bands were mutual between the specimens of two populations. The molecular differences between females and larvae of *T. tabaci* populations propagating on tobacco and on onion resulted in 1 PCR-fragment (1.9%), and 6 ones (11.1%), respectively *(Fig. 1).*

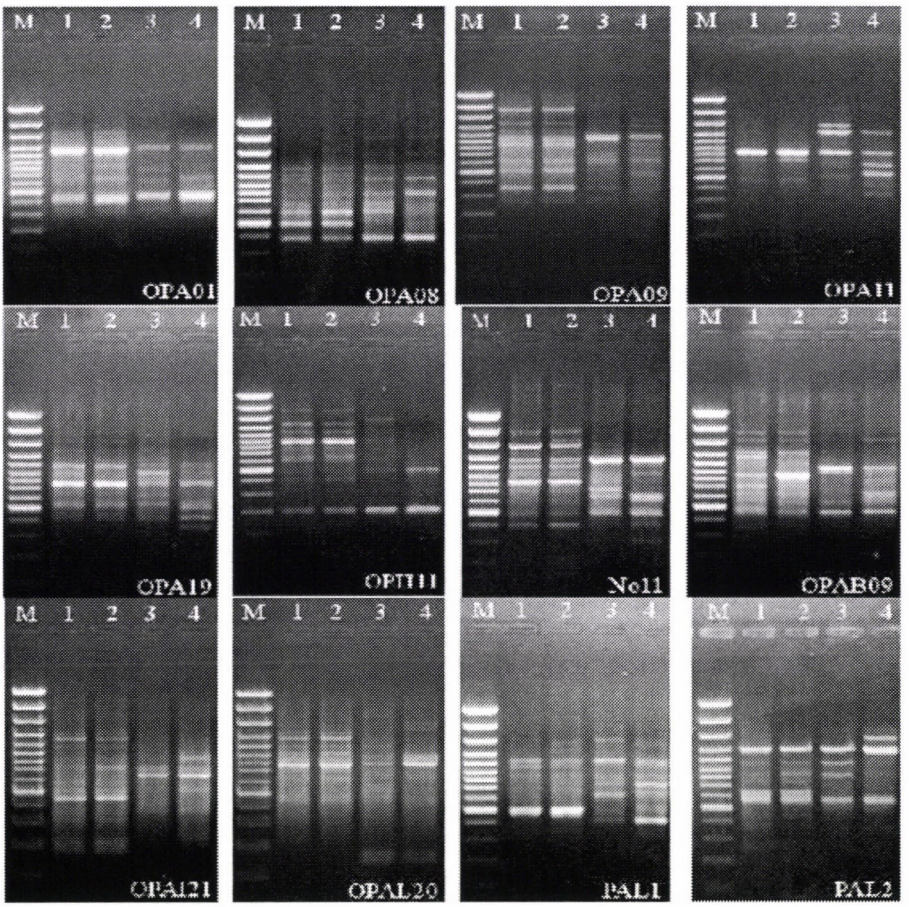


Fig. 1. Twelve samples of agarose gel electrophoresis of PCR amplifications (generated by primers from OPA01 to PAL2) of the populations of *Thrips tabaci* propagating on tobacco or on onion. Lanes, M: Mw, molecular weight marker (arrows show significant Mw fragments in bp); 1 (female adults ♀) and 2 (larvae): *T. tabaci* propagating on tobacco; 3 (female adults ♀) and 4 (larvae): *T. tabaci* propagating on onion

Discussion

According to the available data there are new possibilities in the field of taxonomy both in botany (Gyulai et al., 2000) as well as in entomology in detail in the thysanopterology (Klein and Gafni, 1996; Kraus et al., 1998). Molecular markers such as izoenzyme differences, restriction fragment length polymorphism (RFLP), and especially the PCR-based molecular polymorphisms were found to be helpful to distinguish ambiguous taxa (Klein and Gafni, 1996; Kraus et al., 1998), and also to determine species-specific RAPD-fragments as done in the comparative study of *T. tabaci* and *Frankinellia occidentalis* (Kraus et al., 1998).

In our results presented *T. tabaci* propagating on tobacco showed a characteristic level of molecular differences (72%) compared to those propagating on onion. The significant molecular variations established by these investigations confirmed the opinion of Zawirska (1976) regarding the two *T. tabaci* types (subspecies). Concerning the molecular variations among the *T. tabaci* populations occurring on onion in different agrobiotopes (Klein and Gafni, 1996) as well as the results of the investigations of Kraus and coworkers (1998) and our further investigations are require on *T. tabaci* populations.

Acknowledgements

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Parasitoids and Predators of Coccidae (Homoptera: Coccoidea) Species on Ornamental Plants in Ankara, Turkey

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In this study, the aim of which is to investigate and identify 10 predators, 14 parasitoids and 3 hyperparasitois on 9 different soft scale insects have been identified on ornamental plants in Ankara, Turkey. Predators are: *Anthribus fasciatus* (Först.) (Col. Anthribidae), *Chilocorus bipustulatus* L., *Exochomus quadripustulatus* (L.) (Col.; Coccinellidae), *Cybocephalus fodori minor* E.Y. (Col.: Cybocepalidae), *Pilophorus perplexus* (D.Sc) and *Heterotoma dalmatinum* Wgn (Het.: Miridae), *Leucopis silesiaca* Egger (Dip.: Chamaemyiidae), *Chrysopa* sp. (Neu.: Chrysopidae), *Forficula* sp. (Der.: Forficulidae), Parasitoids; *Coccophagus differens* Jasnosh, *C. lycimnia* (Walker), *C. insidiator* Dalman, *C. piceae* Erdős, *C. proximus* Jasnosh, *C. palaeolecanii* Jasnosh, (Hym.; Aphelinidae), *Metaphychus silvestri* Sugonjaev, *M. dispar* Mercet, *Discodes coccophagus* Ratzeburg, *Blastothrix longipennis* (Howard), *B. hungarica* Erdős, *Aphycoides clavellatus* (Dalman), *Trichomastis* sp. (Hym.; Encyrtidae). In addition to these, *Ceropterocerus mirabilis* Westwood, *Aprostocetus tryapitzini* (Kostjukov), *Pacyneuron muscarum* L. have also been recorded as hyperparasitoid of Coccidae.

Keywords: parasitoids of Coccidae, predators of Coccidae, scale insects.

The ornamental plants play an important role for physical and psychical human health in urban habitats throughout the world. In Turkey, in large cities like Ankara whose population is about 3.5 million, there is currently a growing interest in ornamentals. Expanded use of these plants has caused their cash value to rival and in some instances exceed. On the other hand, ornamental plants are under unsuitable environmental conditions (moisture and nutritional deficiency, higher air temperature, compact soil with poor aeration and occurrence of dust, smoke and gases) as well as many harmful insects. It has been observed that sucking insects, including coccids, are increasing in areas of high air pollution, leading to the mortality of beneficial insects. Mass development of some scale insects in the street plantations is associated not only with indirect effect of abiotic factors but also with direct destruction of entomophages as a results of uncontrolled use of modern pesticides (Kozarzhevskaya, 1987).

In Turkey, the basis for studying taxonomy, morphology, faunistic, biology, economic aspect and natural enemies of coccids were provided mainly by Bodenheimer (1953), Öncüer (1977), Çanakçıoğlu (1977), Özkazanç and Yücel (1985), Yaşar (1990), Ülgentürk and Toros (1998a). Unfortunately, in Turkey, studies on natural enemies of coccids are very few in urban situations except Selmi (1978) and Ülgentürk and Toros (1998b).

In present situation, the control against the harmful insects in the parks and ornamental plants in Ankara is not only difficult, but also inadequate due to insufficient techni-

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cal and theoretical capability of the local governmentel as well as undesired effects of pesticide use on human health. One of the appropriate methods to limit the harmful insects in these environments is biological control. In order to apply this method, both harmful and useful fauna should be well known, the relationships between them should be determined, and if possible useful species should be preserved. From this point of view, this study aims to determine the parasitoid and predators of the Coccidae in urban habitat to provide new data on this subject, and to outline the species that could be used in the biological control.

Materials and Methods

The natural enemies of soft scale insect was surveyed mainly in parks and gardens in Ankara. The samples were collected mainly from twigs infested with scale insects (10–15 cm). The sample collection was made in two different periods. In the first part, the samples were collected biweekly between April to October, then monthly from November to March. Infested twigs and barks was kept under laboratory conditions for the sake of parasitoids. The predator insects were collected directly by shaking the infested branches over a muslin tray. The reared parasitoid and predator specimens were sent for identification.

Results and Discussion

In this study, 10 predators belonging to Coleoptera, Dermaptera, Heteroptera, Neuroptera and 14 parasitoids, 3 hyperparasitoids in Hymenoptera determined on 9 soft scale insects (Coccidae) parks and gardens in Ankara. Soft scale species and their natural enemies are indicated below:

Coccus hesperidum *Linnaeus (Brow soft scale)*

C. hesperidum is commonly found on ornamental plants in greenhouses, houses, and parks. It is determined that this species was found on *Nerium oleander,* and it was parasitized by *Coccophagus lycimmnia* (Walker) (Hym.: Aphelinidae) in greenhouse at Ankara University. It is also noted that the number of parasitized second stage of *C. hesperidum* was very high. This species is known to be preyed by *Scymus rubromaculatus* (Goeze) (Col.: Coccinellidae), and parasitized mainly by *Coccophagus bivittattus* Compere, *C. cowperi* Girault, *C. lycimnia, C. scutellaris* Dalm. (Hym.: Aphelinidae), *Encyrtus lecaniorum* L., *Marietta exitiosa* Compere, *Metaphycus flavus* How. (Hym.: Encyrtidae) (Rosen, 1966; Wood, 1963; Soydanbay, 1976; Öncüer, 1977).

Eulecanium ciliatum *(Douglas) (Ciliate Oak Scale)*

The natural enemies of this species determined by Ülgentürk and Toros (1998b). Thus, in this study only the two species that were not mentioned by them will be discussed. These are: *Pilophorus perplexus* (D.Sc) and *Heterotoma dalmatinum* Wgn. (Het.:

Miridae). Previously parasitoids and predators records of ciliate oak scale are: *Coccophagus aterrimus* Vikberg (Hym.; Aphelinidae), *Encyrtus albitarsus* Zett, *E. infidus* (Rossi), *Metaphycus dispar* Mercet, *Microterys hortulans* Erdős, *Psyllaephagus procerus* (Mercet) (Hym.; Encyrtidae), *Enotus areolatus* (Ratzeburg) (Hym.; Pteromalidae), *Anthribus fasciatus* (Först.) (Col.: Anthribidae) *Adalia bipunctata* L., *A. fasciatopunctata revelierei* Muls. *Chilocorus bipustulatus* L., *Exochomus quadripustulatus* (L.), *Synharmonia conglabata* L. (Col.: Coccinellidae), *Cybocephalus fodori minor* E. Y. (Col.: Cybocephalidae) *Atractotomus mali* (Meyer-Dür), *Deraecoris ruber* (Lineé) (Het.: Miridae), (Ülgentürk and Toros, 1998b).

Eulecanium tiliae *(Linnaeus) (Nut Scale)*

This species is common but usually in specimens on *Betula* sp., *Rosa* sp., *Pyrus vulgaris, Ulmus campestre.* During this study *Anthribus fasciatus* were determined as predator, and *Metaphycus dispar* were determined as parasitoid of Nut scale.

Some of the known parasitoids of *E. tiliae* are the encyrtids *Blastothrix longipennis* (Howard), *B. sericea* Dalman, *Chelineuros claviger* Thompson, *Encyrtus infidus, E. lecaniorum, Metaphycus dispar, M. sylvius* Sugonyaev, *Microterys aeneiventris, M. lunatus* (Dalman), aphelinids; *Coccophagus lycimmnia, C. scutellaris.* The beetle *Anthribus fasciatus, A. nebulosus* Forster and *Chilocorus bipustulatus* has been reported as predators (Rubin and Beirne, 1975; Sugonyaev, 1976; Cannard, 1980; Ülgentürk and Toros, 1996).

Filippia follicularis *(Targioni Tozzetti)*

F. follicularis, found generally within the neglected olive groves of Turkey, is observed in large proportions in association with *Fraxinus americana F. excelsa* in Ankara (Ülgentürk and Toros, 1998a). Of these, three predators, two parasitoids, and one hyperparasitoid were distinguished *(Table 1).*

Table 1

Predators and parasitoids of *Filippia follicularis* (Targioni Tozzetti) in Ankara parks and gardens

Species	Order/Family
Predators	
Exochomus quadripustulatus (L.)	Coleoptera : Coccinellidae
Leucopis silesiaca Egger	Diptera : Chamaemyiidae
Chrysopa sp.	Neuroptera : Chrysopidae
Parasitoids	
Metaphycus dispar Mercet	Hymenoptera : Encyrtidae
Blastothrix hungarica Erdős	„
Pachneuron muscarum L.*	Hymenoptera: Pteromalidae

*Hyperparasitoid

Previous records of predators on *Filippia follicularis* are: *Exochomus quadripustulatus* and *Leucospis silesiaca* Egger (Öncüer,1977; Longo, 1986; Raspi and Bertolini, 1993; Ülgentürk, 1999). Also, the parasitoids of this species are: *Cheiloneurus claviger, Microterys lunatus, M. masii* (Silv.) (Hym.; Encyrtidae) (Öncüer, 1977; Yayla, 1983).

Palaeolecanium bituberculatum *(Targioni-Tozzetti) (Bituberculate Scale)*

P. bituberculatum is harmful on *Crateagus* sp., *Malus* sp. and *Rosa* sp. in Ankara parks and gardens. In this study, the parasitoids of this species are not obtained, although rarely parasitized individuals are observed. But, *Adalia fasciatopuctata revelierei* feeding by I and II stage nymphs of *P. bituberculatum* on the leaves, were found. *Coccophagus differens* Jasnosh, *C. lycimnia, C. insidiator* Dalman, *C. palaeolecanii* Jasnosh, *C. scutellaris, Microterys duplicatus* (Nees), and *Trichomasthus albimanus* Thomson are distinguished as parasitoids of this species (Trjapitzin, 1978; Erol and Yaşar, 1996).

Parthenolecanium rufulum *(Cockerell) (Oak Soft Scale)*

Two parasitoids were identified on *P. rufulum,* these are *Trichomasthus* sp. and *Blastothrix hungarica* Erdős from Encyrtidae. Trjapitzin (1978) is noted parasitoids in this species: *Blastothrix confusa* Erdős, *Coccophagus lycimnia* and *Metaphycus insidiosus* Mercet.

Physokermes piceae *(Schrank) (Spruce-Bud Scale)*

P. piceae is very common on *Piceae excelsa, P. excelsa* var. *maxvellii, P. orientalis, P. pungens, P. pungens* var. *glauca* in Ankara parks and gardens. A great number of parasitoid and predator was obtained from *P. piceae* samples from these hosts. These are: 2 coccinellid, 1 dermapter predator, 2 aphelinid, 2 encyrtid parasitoid and 1 pteromalid hyperparasitoid *(Table 2).*

Table 2

Predators and parasitoids of *Physokermes piceae* (Schrank) on ornamental plant in Ankara

Species	Order/Family
Predators	
Chilocorus bipustulatus L.	Coleoptera : Coccinellidae
Exochomus quadripustulatus (L.)	"
Forficula sp.	Dermaptera : Forficulidae
Parasitoids	
Coccophagus insidiator Dalman	Hymenoptera : Aphelinidae
C. piceae Erdős	Hymenoptera : Aphelidae
Aphycoides clavellatus (Dalman)	Hymenoptera : Encyrtidae
Trichomastis sp.	"
Pacyneuron muscarum L.*	Hymenoptera : Pteromalidae

*Hyperparasitoid

The natural enemies of this species is not known in Turkey. However, it is argued that it is parasitized by *Aphycoides clavellatus* (Dalman), *Chelineurus formosus* (Boheman) (Hymenoptera: Encyrtidae), *Coccophagus insidiator, C. lycimmnia, Microterys fuscipennis* Dalman, *M. lunatus* and they are the predators of species like *Exochomus quadripustulatus* and *Scymus abietis* Paykull (Schmutterer, 1965; Sugonyaev, 1976; Trjapitzin, 1978).

Shpaerolecanium prunastri *(Fonscolombe) (Plum lecanium)*

S. prunastri which is one of the most effective harmful species of the stone fruits, is very common within the ornamental plum in Ankara parks. In this study 2 coccinellid predators, 6 parasitoids, and 3 hyperparazitoids were obtained from the *S. prunastri* samples on *Prunus domestica, P. cerasifera, P. cerasifera* var. *pissardi (Table 3).*

Table 3

Predators and parasitoids of *Sphaerolecanium prunastri* Fonsc. on ornamental plants in Ankara

Species	Order/Family
Predators	
Chilocorus bipustulatus L.	Coleoptera : Coccinellidae
Exochomus quadripustulatus (L.)	"
Cybocephalus fodori minor E. Y.	Coleoptera : Cybocephalidae
Parasitoids	
Coccophagus differens Jasnosh	Hymenoptera : Aphelinidae
C. proximus Jasnosh	"
C. sp. nr palaeolecanii Jasnosh	"
Metaphycus silvestri Sugonjaev	Hymenoptera : Encyrtidae
Blastothrix longipennis Dalman	Hymenoptera : Encyrtidae
Discodes coccophagus Ratz	"
Ceropterocerus mirabilis Westwood*	"
Aprostocetus tryapitzini (Kostjukov)*	Hymenoptera : Eulophidae
Pacyneuron muscarum L.*	Hymenoptera : Pteromalidae

*Hyperparasitoid

The parasitoids of *S. prunastri* distinguished in Turkey are *Coccophagus lycimmnia, C. scutellaris, Discodes aeneus* (Dalman), *D. coccophagus* Ratz. *Metaphycus dispar, M. insidiosus* Mercet, and *Microterys* sp. (Gürkan, 1974; Soydanbay, 1976; Öncüer, 1977). The predators of this species are *Orius minutus* (L.) (Het.; Anthocoridae), *Adalia bipunctata, Exochomus quadripustulatus, Scymnus apetsi* (Muls.), *S. interruptus* (Goezze), *Synharmonia conglabata* and *Cybocephalus fodori* E. Y. (Ben-Dov, 1968; Gürkan, 1974; Soydanbay, 1976; Öncüer, 1977; Kozar et al., 1982; Bakogiannis, 1984; Moglan, 1995).

The result of this study is understood that very rich complex of natural enemies of soft scale insects on ornamental plants in Ankara. Ten predators (3 coccinellid, 2 mirid and once of each antribid, cybocephalid, chamaemyiid, chrysopid, forficulid), 14 parasitoids (7 aphelinid, 7 encyrtid) and 3 hyperparasitoids have been found in this habitat. *Exochomus quadripustulatus* which is polyphagous, their larvae was preyed eggs and larvae of *Filippia*

follicularis, Physokermes piceae and *Sphaerolecanium prunastri.* Anthribid beetle *Anthribus fasciatus* is commonly found as eggs predator on *Eulecanium ciliatum* and *E. tiliae.* This predator has completed all life cycle in one coccids female. For this reason, it works slowly but effectively (Cannard, 1980; Ülgentürk and Toros, 1996). *A. fasciatus* Först. (Col.; Anthribidae) were found in 73% of *E. ciliatum* (Ülgentürk and Toros, 1998b).

In this study, every parasitoid species is found on only one coccid species but hyperparasitoid *Pacyneuron muscarum* is found *F. follicularis, P. piceae* and *S. prunastri.*

It is certain that these natural enemies are very effective for control on soft scale insect, although it is not enough. With public opinion becoming more sensitive to the hazards of human exposure to toxic chemicals, there is a very real need to conduct extensive research on methods of exploiting alternative control techniques and using insecticides only when absolutely essential. This need is especially important in ornamental situations, since humans are in frequent contact with the plants in this habitat. The IPM approach is an obvious solution to the problem, but it requires detailed information on all components that impinge on the pest system. These data simply are not available for most soft scale insects. It is surprising how little is known about even some major coccoid pest, let alone those that are considered minor (Davidson and Miller, 1990). Data on such things as life history, natural enemies, host range, host preference, ecology are critical but often are unknown. It is apparent that considerable effort must be brougt to bear on this truly challenging area of research.

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Initial Studies on Pseudococcidae Fauna in Van Province of Turkey

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This study was carried out at Van province, Turkey between 1996 and 1998. Wild plants and cultivated crops from different habitats were surveyed and mealybug samples were collected. Slides were prepared from these samples and nineteen species were identified of which 2 have been previously identified. These species were found belonging to 8 genera which are belong to the family Pseudococcidae. *Antonina graminis, Atrococcus ingidens, Atrococcus saxatilis, Euripersia amnicola, Heliococcus bohemicus, Heliococcus saxatilis, Mirococcus inermis, Peliococcus bitubulatus, Peliococcus manifectus, Peliococcus terrestris, Peliococcus turanicus, Peliococcus unispinus, Phenacoccus affinis, Phenacoccus karaberdi, Phenacoccus tergrigorianae, Trionymus abberans* and *Trionymus graminellus* are for the first time to be recorded in Turkey.

Keywords: Homoptera, Coccoidea, Pseudococcidae, Turkey, distribution, host plants.

Due to geographical situation, Turkey has different climatic regions which are rich in flora and fauna. Zoogeographically Turkey lays among Iran-Turano, Mediterranean and Euro-Siberian subregions of Palaearctic region. Pseudococcidae fauna was quite well studied in the world. However, in Turkey, it is not well studied. The study of this family was started by Bodenheimer (1953a, b). Also it was studied by Çanakçıoğlu (1977) who identified 3 species in forestry areas, but the most intensive study was conducted by Düzgüneş (1982) where 13 mealybug species were determined.

This study was conducted in Van province in east Anatolia region of Turkey *(Fig. 1)*. This geographic part consists of the area around Van lake which includes some mountainous parts. The inland climate is characterised by long cold and snowy winters, and hot and dry summers. The whole area dominated by higher elevations over 1700 m above sea level. Similar study was conducted by Ter-Grigorian (1973) in Armenia which is the neighbouring area to this region. The aim of this study to identify the different species of the Pseudococcidae family pervading in Van province of Turkey.

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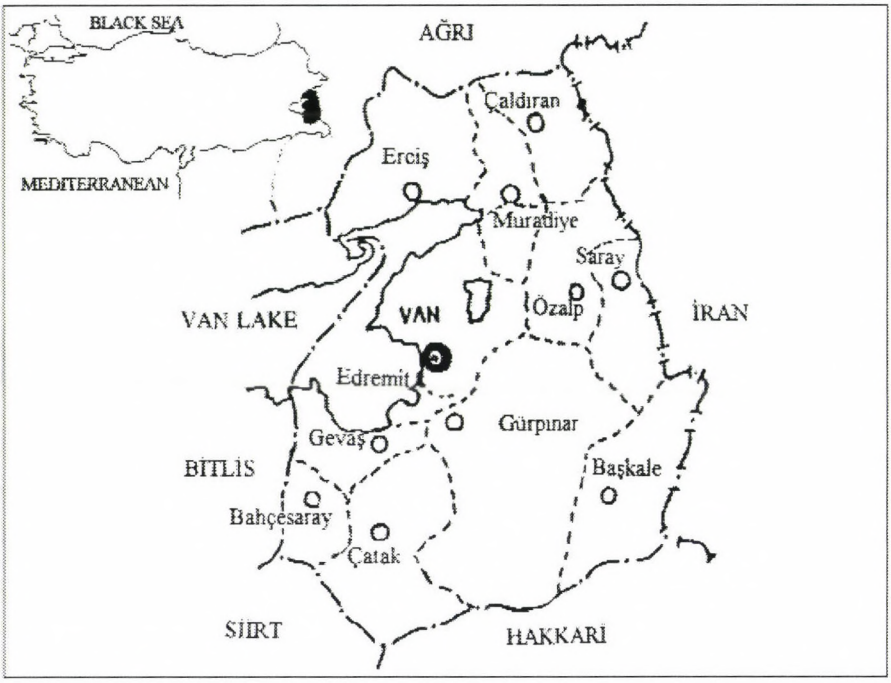


Fig. 1. The map of Van province of Turkey

Materials and Methods

Samples were collected from Van province of Turkey between 1996–98. The samples were collected from 36 plant species belonging to 13 families in 11 different sites.

Description of samples contains the names of identified mealybug species, place of collection, host plant and collection date. Parts of dry and mounted material are deposited at the Plant Protection Department of Yüzüncü Yıl University in Van, Turkey.

Results and Discussion

Totally 19 species of mealybugs were found in Van province in this study. Seventeen of them were newly recorded in Turkish mealybug fauna. The number of mealybug species known in Turkey increase to 33 species. Ter-Grigorian (1973) and Borchsenius (1949) cited 84 species from similar geographic area. As to the zoogeographical distribution of the species involved, the majority is Palaearctic, others are cosmopolite. This may necessitate detail faunistic studies particularly in this region which have not been fully studied up to now.

List of collected species with host plant and phonological data:

Antonina graminis (Maskell, 1897)

Material examined: Van (around the lake area), *Lolium multiflorum,* 03. 08. 1997; First record in Turkey.

This genus is usually found on clumps or under leaf sheaths of Gramineae plants. Sixteen species in the world (Ben-Dov, 1994) and 9 species in Palaearctic region (Kozár, 1998) were recorded. One other species *A. bambusa* was recorded at Rize in Turkey (Bodenheimer, 1953a, b; Düzgüneş, 1982).

Atrococcus ingidens (Borchsenius, 1949)

Material examined: Van (around the lake area); *Astragalus* sp., 03. 08. 1997. First record in Turkey.

Atracoccus saxatilis (Ter-Grigorian, 1964)

Material examined: Özalp, *Salvia* sp. roots 17. 06. 1997. First record in Turkey.

Beside perennial plants, trees are also serve as hosts for this genus. Only 2 species had been recorded in Turkey.

Euripersia amnicola (Borchsenius,1948)

Material examined: Özalp, *Stipa holosenica* roots 03. 07. 1997. First record in Turkey.

Eupersia genus usually found on roots of Gramineae species. There are 19 species in the world (Ben-Dov, 1994) of which 17 species in the Palaearctic region (Kozár, 1998).

Heliococcus bohemicus (Sulc, 1912)

Material examined: Gürpınar, *Pholomis* sp. 16. 07. 1997. First record in Turkey.

Heliococcus saxatilis (Borchsenius, 1949)

Material examined: Çatak, *Nepeta* sp., 25. 07. 1997. First record in Turkey.

Heliococcus species are mostly polyphagus which could be found on trees and perennial plants roots, stems, branches and leaves. There are 66 species in the world (Ben-Dov, 1994) of which 56 species in the Palaearctic region (Kozár, 1998).

Mirococcus inermis (Hall, 1925)

Material examined: Van Yüzüncü Yıl University Campus, *Chenopodium album, Heliotropium europaeum, Polygonum* sp. roots, 08. 07. 1996; Hoşap, *Salsola kali* roots 16. 08. 1996. First record in Turkey.

Mirococcus genus is usually found on dicotyledoneus plants roots and under leaf sheaths of grasses (Kosztarab and Kozár, 1988). This genus contains 8 species in Palaearctic region and the world (Ben-Dov, 1994; Kozár, 1998).

Peliococcus bitubulatus (Borchsenius, 1949)

Material examined: Van Yüzüncü Yıl Universty Campus, *Cuminum cyminum* roots, 31. 07. 1997. First record in Turkey.

Peliococcus chersonensis (Kiritchenko, 1935)

Material examined: Ercis, One of the Umbelliferae species 16. 07. 1997; Çatak, *Cnopis* sp., *Globularia* sp. roots, 25. 07. 1997.

This species was recorded from Ankara (Bala) on the roots of *Artemisia fragrans* by Bodenheimer (1953).

Peliococcus manifectus (Borchsenius, 1949)

Material examined: Muradiye, *Euphorbia* sp. roots 21. 08. 1996; Saray, *Turgenia latifolia* roots, 02. 08. 1997. First record in Turkey.

Peliococcus terrestris (Borchsenius, 1949)

Material examined: Başkale, *Euphorbia* sp. roots 16. 08. 1997; Saray, *Euphorbia* sp. roots 02. 08. 1997. First record in Turkey.

Peliococcus turanicus (Kiritchenko, 1932)

Material examined: Gevaş, *Achillea* sp. roots 09. 07.1 996; Erciş, *Euphorbia* sp. roots 05. 08. 1997; Saray, *Tragopogon* sp., *Falcaria vulgaris, Senecio* sp. *Cardaria draba* roots 02. 08. 1997. First record in Turkey.

Peliococcus unispinus (Borchsenius and Ter-Grigorian, 1956)

Material examined: Saray, *Turgenia latifolia* roots, 02. 08. 1997. First record in Turkey.

Peliococcus genus is usually found on roots and foliage of perennial plants, and occasionally found on branches of woody plants (Kosztarab and Kozár, 1988). There are 52 species in the world (Ben-Dov, 1994) of which 43 species in the Palaearctic region (Kozár, 1998). In previous studies only 1 species was recorded.

Phenacoccus affinis (Ter-Grigorian, 1963)

Material examined: Muradiye, *Beta vulgaris* roots, 24. 07. 1997. First record in Turkey.

Phenacoccus karaberdi (Borchenius and Ter-Grigorian, 1956)

Material examined: Saray, *Tiriticum vulgare* leaf sheaths, 02. 08. 1997. First record in Turkey.

Phenacoccus tergrigorianae (Borchsenius and Ter-Grigorian, 1956)

Material examined: Van Yüzüncü Yıl University Campus, *Chenopodium* sp. 08.

07. 1996; Gevaş, *Chenopodium album,* 09. 07. 1996; Erciş, *Umbelliferae,* 16. 07. 1996, 05. 08. 1996; *Cichorium* sp., 17. 08. 1996; Muradiye, *Beta vulgaris,* 21. 08. 1996; Özalp, *Zygophyllum tabacum,* 03. 07. 1997; Campus, *Chenopodium* sp., 08. 07. 1996; Campus, *Onobrychis vitifolia,* 04. 07. 1996, 08. 07. 1996; Gevaş, *Hordeum vulgare,* 16. 07. 1997; Gürpınar, *Melilotus* sp. roots 16. 07. 1997. First record in Turkey.

Phenacoccus genus is found on roots and leaves on both woody and perennial plants (Kosztarab and Kozár, 1988). Most of the species are cosmopolitan which are distributed all over the World. This genus has 175 species in the World (Ben-Dov, 1994) of which 114 species in the Palaearctic region (Kozár, 1998). 4 species of *Phenacoccus* genus, were recorded before in Turkey.

Trionymus abberans (Goux, 1938)

Material examined: Gevaş, *Elymus repens* 16. 07. 1997. First record in Turkey.

Trionymus graminellus (Borchsenius, 1949)

Material examined: Çatak, *Caucalis ptatycorpis* roots, 25. 07. 1997. First record in Turkey.

Trionymus multivorus (Kritchenko, 1936)

Material examined: Gevaş, *Medicago sativa* 16. 07. 1997; Çatak, *Daucus guttatus, Bunium* sp., *Nepeta* sp., 25. 07. 1997. This species had been recorded by Bodenheimer (1953a) and Çanakçıoğlu (1977) as *Pseudococcus multivorus.*

Trionymus genus is usually found on grass leaves, under the leaf sheaths, stems and roots. Only *T. newsteadi* is found on trees. This genus could be found all over the World (Kosztarab and Kozár, 1988). It has 120 species in the World (Ben-Dov, 1994) and 62 species in Palaearctic region (Kozár, 1998). Only 1 species was recorded by Çanakçıoğlu (1977) in Turkey.

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Effect of *Tagetes erecta* on the Mortality, Hatching and Development of *Meloidogyne incognita* (Kofoid and White) Chitwood

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Effect of different aqueous extracts of *Tagetes erecta* on the mortality, hatching and of *M. incognita* varied with concentration, period of exposure and nature of extracts. The greatest percentage of mortality and inhibition hatching of *M. incognita* juvenil was observed in roots and mixture of different parts extracts with respectively 62, 71% and 63, 67%. Interculture of *Tagetes erecta* with tomato reduce also the multiplication of *Meloidogyne incognita* on tomato.

Keywords: tomato, *Tagetes erecta,* aqueous extracts, *Meloidogyne incognita,* mortality, hatching, development, interculture.

Vegetable crops has widely spread all over the country during the last decade particulary those set in greenhouses. The total area covered by such cropping technique was 5000 ha in 1995 (Anonyme, 1996). However, the intensive use of this type of crop transformated the spread of many pests such as the nematode. Among these, Root–knot nematode *Meloidogyne* is an important pest also in field conditions than in greenhouses in Algeria (Lamberti et al., 1975; Sellami et al., 1999). Aviable nematicides are too costly for use on most crop, and they be dangerous to the public health. These last years various plant have been reported to have a nematicidal or nemastatic nematodes properties (Gommers, 1981; Hussain et al., 1984; Ketel, 1986; Grainge and Ahmed, 1988). Among these plants, *Tagetes* seems to be promotor, several studies have been shown effects of Tagetes to reduce phytonematode populations (Siddiqui and Alam, 1987; Siddiqui and Alam, 1988; Cayrol, 1991; Conijn, 1994; Owino et al., 1996). The present study was undertaken to investigate the effects of different plant parts aqueous extracts of *Tagetes erecta* on the mortality, hatching of juveniles *M. incognita* and study of growing of *Tagetes erecta* intermixed with tomato for controlling this nematode.

Materials and Methods

The aqueous extracts were prepared by drying and grinding roots, leaves, flowers separately and different plant parts (mixture) in distilled water. They were filtered through Whatman filter paper then centrifuged. The solution thus obtained was designed as standard (S). S/2 and S/5 dilutions were also made from the standard (S). For juvenile mortality studies, 100 freshly hached second stage juveniles of *Meloidogyne incognita* were transfer-

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ed to Petri dishes (5 cm diameter) containing 5 ml of aqueous extracts. Distilled water and Ethoprophos 10% (50 kg ia/ha) were used as control. For each treatment three replicates were realised. Dead and survival nematodes were counted after 24, 48, 72 hours the mean percentage mortality was calculated.

The effect of the aqueous extracts on the juvenile hatching of *M. incognita,* was also investigated. One sized and freshly picked eggmasse was transferred to Petri dishes (5 cm diameter) containing 5 ml of different dilutions of the aqueous extracts. There were five replicates for each treatment including the control. The total number of hatched juveniles was counted after 8 days and percent inhibition of the juveniles hatching was calculated.

The effect of *Tagetes erecta* as interculture plant controlling *M. incognita* was studied in pots. Three weeks old seedlings of tomato *(Lycopersicon esculentum* Mill cv Tango) were transplanted singly into the center of pots containing 2 kg autoclaved mixture of soil, sand loam and compost, each plant was inoculated with 2500 freshly second juveniles (J2) of *M. incognita,* one, three and five seedlings of 20-day-old *Tagetes* erecta were transplanted into each pot. For each treatment five replicates were realised, control was represented with tomato but without *Tagetes.* The pots were arranged in a randomized complete block. The experiment was maintened in a glasshouse at 25±2 °C. After three months, plant growth was determined by mesuaring shoot height, Root-knot gall index was rated (0–5 scale). Final population in the soil were determined by decanting method with modified Baermann funnels technique.

Results and Discussion

Results presented in *Table 1* indicate that juveniles mortality increase in the concentration, nature extracts and the exposure period. (S) concentration proved highly toxic but in lower concentrations the toxic effect was noticeable excepted with different plant parts (mixture). The present findings confirm the studies of Siddiqui and Alam (1987) who reported the nematicide nature of root exudates of *Tagetes minuta* with a percentage of mortality about 60% against *M. incognita,* this percentage was more high against *Rotylenchus reniformis* and *Tylenchorhynchus brassicae.*

Data on the hatching eggs after 8th days revealed that the inhibition of hatching was maximum in S and S/2 of roots and mixture extracts followed by S. of flowers. The respective percentage of inhibition over control were 63, 67%. The other dilutions recorded less than 50% inhibition *(Table 2).* The roots and mixture extracts were more effective than the others. Nemadicidal activity was the same as that Ethoprophos. These findings were in agreement with those previous authors who reported that the inhibition of juvenile hatching from eggmasses of *M. incognita* exposed to the root exudate for 5 days was 80% at the high concentration (S) (Siddiqui and Alam, 1987).

Results from intermixture of *Tagetes* with tomato indicate that as the number of *T. erecta* plants/pots was increased, the numbers of root galls and nematodes in the soil decreased *(Table 3).* Treatments with five plants of *T. erecta* caused the greatest reduction in the root gall index and nematode populations. In addition to this, the growth of the plants

Table 1

Effect of aqueous extracts of different plant parts on the mortality of juveniles of *Meloidogyne incognita*

Plant extract	Time of exposure (hours)	Percentage of mortality at differents concentrations*		
		S	S/2	S/5
Tagetes erecta (leaves)	24	20	18	6
	48	27	21	14
	72	48	39	20
Tagetes erecta (roots)	24	31	28	14
	48	52	34	20
	72	62	48	28
Tagetes erecta (flowers)	24	24	22	7
	48	32	26	14
	72	51	40	23
Tagetes erecta (mixture)	24	35	30	21
	48	55	38	32
	72	71	49	33
Ethoprophos (control)	24		49	
	48		60	
	72		79	
Distilled water (control)	24		0	
	48		0	
	72		1	

*Each value is a mean of three replicates

Table 2

Effect of aqueous extracts on the juvenile hatching of *Meloidogyne incognita*

Plant extract	Number of juveniles hatched after 8 days			% inhibition in hatching over control		
	S	S/2	S/5	S	S/2	S/5
Tagetes erecta (leaves)	200.4	240.2	297.1	45.70	34.80	20
Tagetes erecta (roots)	136.2	199.8	235.2	63.20	45.83	36.15
Tagetes erecta (flowers)	156.6	215.6	277.1	57.60	41.58	24.90
Tagetes erecta (mixture)	122.2	149.5	184.5	67	59.70	50.2
Ethoprophos (control)		142.2			61.5	
Distilled water (control)		368.8			–	

Table 3

Effect of aqueous extracts of different plant parts on the mortality of juveniles of *Meloidogyne incognita*

Treatments	Length (cm)	Root-Knot index	Final population	R=Pf/Pi	% decrease over control
Tomato + 1*Tagetes*	33	3	4556	1.79	16.90
Tomato + 3*Tagetes*	36.85	2.75	3246	1.25	40.80
Tomato + 5*Tagetes*	43.25	1.5	1939	0.77	64.70
Ethoprophos (control)	43.75	1.8	1986	0.79	63.80
Tomato without *Tagetes*	32.77	3.75	5483	2.19	–

Each value is a mean of five repetition
R = Reproduction factor
Pi = Initial population
Pf = Final population

also improved with a percentage of 32%. These results are in agreement with those of previous authors (Alam et al., 1977; Siddiqui and Alam, 1987). Also, incorporation of crop residues of *Tagetes erecta* reduced 88% populations of *M. incognita* in pepper and fruit yield was increased 37.6% (Zavaleta-Mejia et al., 1993).

It is evident from these results that *Tagetes erecta* even in crude water extracts posses nematicidal properties. When nematode enter into the roots they killed by a reaction terthienieyl and related compounds (Gommers and Bakker, 1988). Thiophenes have been characterized in different cultivated species of Tagetes (Gommers, 1981; Ketel, 1986; Tang et al., 1986). The compounds derived from 2,2-bithienyl presented a good nematicidal activity. Others compounds as: 2,3'-4,2''-thienyls and also several methyl polythienyls present in *Tagetes* (*T. erecta* and *T. patula*) were revealed also very efficient (Uhlenbroek and Bijloo, 1960). Recently, the presence of the biocidal metabolites has been reported and the best known thiophenes were present in leaves and roots of both *Tagetes mendocina* and *Tagetes argentina* (Benavides and Caso, 1992).

Hypothese that diffusates from *Tagetes* spp might supply neutralize or mask host diffusates making orientation to roots difficult was also reported by Christie (1960). These results show that *Tagetes* has the similar effect than Ethoprophos and may offer an alternative method of nematode control. Use of this plant parasitic has a great interest because of effectiveness, minimal phytotoxicity, less expensive than nematicides and essensialy easily avaibale for agricultor. However, further investigations are required in field conditions to ascertain whether this plant can be effectively used for control nematode.

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An Annotated List of Scale Insects (Homoptera: Coccoidea) from Turkey

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A list of 176 species of Coccoidea recorded from Turkey, belonging to 13 families and 84 genus, is presented. This list is taken from the authors collection records and from bibliographical sources. The most numerous families are Diaspididae (93 species), Coccidae (33 species) and Pseudococcidae (22 species). The remaining families have between 1 and 6 species each. Of the 176 species 12 are considered to be endemic. In Turkish orchard systems, 15 species are of sufficient economic importance to require control measures. In addition to the cultivated plants, non-cultivated plants have many scale insect on them because of the zoogeographical situation of Turkey, lying as it does between the Mediterranean, the Irano-Turanian and Euro-Siberian subregions of Palaearctic. This list is unlikely to be complete and further studies are needed.

Keywords: Homoptera, Coccoidea, scale insects.

Throughout the world, scale insects have been recorded from most climatic regions particularly on perennial crops, woody plants, ornamentals, forests and macchia. Those of Turkey are only poorly known and the most detailed studies have been made by the following authors: Bodenheimer (1949, 1952, 1953a, 1953b), Öncüer (1974, 1977), Çanakcıoğlu (1977), Kozar et al. (1979), Selmi (1979), Düzgüneş (1982), Lodos (1986), Yaşar (1995), Erler et al. (1996) and Uygun et al. (1998). Additional records could be found about distribution and biology of some species in the next publications: Ben-Dov (1990, 1994), Çiftci (1982), Çanakcıoğlu (1994), Çobanoğlu and Düzgüneş (1984), Danzig and Miller (1996), Düzgüneş and Özkök (1940), Düzgüneş et al. (1975), Erkılıç and Uygun (1995, Eronç (1971), Erdem (1976), Erkam (1981), Gül-Zümreoğlu (1972), Gürkan (1982), Hoy (1963), Kıroğlu (1981), Kosztarab and Kozár (1988), Kozár (1995, 1998), Lindinger (1912), Okul et al. (1983), Önder (1982), Soylu (1976), Williams and Miller (1985), Yaşar (1990), Yaşar et al. (1995).

Turkey has been divided in seven geographically different regions namely, Marmara region (Mar. R.), Blacksea region (B. R.), Aegean region (A. R.), Central Anatolia region (C. A. R.), Mediterrannean region (Med. R.), East Anatolia region (E. A. R.) and Southeast Anatolia region (S. A. R.) *(Table 1)*. These regions show different climatic features, such as Marmara, Blacksea, Aegean and Mediterranean regions fairly mild winters compare to cold winters of Central, East and Southeast Anatolia regions due to sea effect around these regions. Mediterranean region shown the typical Mediterranean type of climate, rainy winters but dry summers. The other regions which have typical inland climate with long

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Table 1

The list of scale insects determined in Turkey, the host plants and zoogeographical distribution

	Host plants	Zoogeographical region	Distribution in Turkey
ASTEROLECANIIDAE			
001. *Asterodiaspis*			
1. *ilicicola* (Targioni-Tozzetti)	*Q. coccifera, Quercus* spp.	Mediterranean	Med. R., A. R.
2. *quercicola* (Bouche)	*Q. branti, Quercus* spp.	Cosmopolit	C. A. R., Mar, R., Med. R., A. R.
3. *variolosa* (Ratzeburg)	*Quercus aegilops, Q. coccifera*	Cosmopolit	A. R.
002. *Planchonia*			
1. *arabidis* Signoret	_	Cosmopolit	_
2. *zanthenes* (Russel)	_	Palaearctic	_
CEROCOCCIDAE			
003. *Cerococcus*			
1. *perowskiae* Archangelskaia	_	Palaearctic	_
004. *Pollinia*			
1. *pollini* (Costa)	*Q. europaea, Olea* spp.	Mediterranean	Med. R., A. R., S. A. R.
COCCIDAE			
005. *Anapulvinaria*			
1. *pistaciae* (Bodenheimer)	*Pistacia atlantica, P. vera*	Mediterranean, Irano-Turanian	S. A. R., Mar. R.
006. *Bodenheimera*			
1. *rachelae* (Bodenheimer)	*Vitex agnus castus*	Mediterranean, Irano-Turanian	Med. R.
007. *Ceroplastes*			
1. *floridensis* Comstock	Polyphag on fruit trees and ornamentals	Cosmopolit	Med. R., A. R.
2. *rusci* (Linnaeus)	Polyphag on fruit trees and ornamentals	Cosmopolit	Med. R., A. R.
3. *sinensis* Del Guercio	Polyphag on fruit trees and ornamentals	Cosmopolit	A. R., B. R.
008. *Chloropulvinaria*			
1. *floccifera* (Westwood)	Polyphag on fruit trees and ornamentals	Cosmopolit	Med. R., B. R., Mar. R.
009. *Coccus*			
1. *hesperidum* Linnaeus	Polyphag on fruit trees and ornamentals	Cosmopolit	Med. R., C. A. R., A. R.,Mar. R.,
2. *pseudomagnoliarum* (Kuwana)	Polyphag on fruit trees and ornamentals	Cosmopolit	Med. R., C. A. R., A. R., Mar. R.,
010. *Eriopeltis*			
1. *festucae* (Fonscolombe)	*Alopecurus agrestis, Agropyrum* sp.	Nearctic, Palaearctic	C. A. R.

Table 1 (cont.)

	Host plants	Zoogeographical region	Distribution in Turkey
011. *Eucalymnatus*			
1. *tessellatus* (Signoret)	_	Cosmopolit	C. A. R.
012. *Eulecanium*			
1. *ciliatum* (Douglas)	*Jasminum fruticans*	Palaearctic	C. A. R.
2. *kosswigi* Bodenheimer	_	Palaearctic	C. A. R.
3. *pistaciae* Borchsenius	_	Palaearctic	–
4. *rugulosum* (Archangelskala)	*Prunus persica*	Palaearctic	Med. R.
5. *takachihoi* (Kuwana)	*Vitex agnus castus*	Palaearctic	Med. R.
6. *tiliae* (Linnaeus)	Polyphag on fruit trees and ornamentals	Oriental, Nearctic, Palaearctic	C. A. R., Mar. R., Med. R., B. R.
7. *transvittatum* Green	*Acer negunda*	Palaearctic	C. A. R.
013. *Eupulvinaria*			
1. *citricola* Kuwana	_	Oriental, Nearctic, Palaearctic	–
014. *Filippia*			
1. *follicularis* Targioni-Tozzetti	*Olea* spp.	Palaearctic	Mar. R., A. R.
2. *oleae* (Costa)	*O. europaea, Olea* spp., *Viburnum* sp.	Palaearctic	A. R., Mar. R.
015. *Lecanopsis*			
1. *turcica* (Bodenheimer)	_	Palaearctic	C. A. R.
016. Nemolecanium			
1. *apti* (Bodenheimer)	*Abies nordmanniana*	ENDEMIC	B. R.
017. *Palaeolecanium*			
1. *bituberculatum* (Signoret)	Stone and pome fruits, and *Pyrus elaeagnifolia*	Palaearctic	C. A. R., Med. R., S. A. R.
018. Parthenolecanium			
1. *corni* (Bouché)	Stone and pome fruits, *Morus alba, Prunus armenica*	Cosmopolit	C. A. R., B. R., Mar. R., A. R.
2. *persicae* (Fabricius)	*Vitis vinifera, Morus alba, Prunus persica, Elaeganus* sp.	Cosmopolit	
3. *pomeranicum* (Kaweckl)	*Taxus baccata*	Palaearctic	Mar. R.
4. *pruinosum* Coquillett	–	Palaearctic, Nearctic	–
5. *rufulum* (Cockerell)	*Betulaceae, Fagaceae, Leguminosae, Rosaceae*	Palaearctic	C. A. R., Mar. R.
6. *tamarics* (Bodenheimer)	*Tamarix pallasii*	ENDEMIC	C. A. R.
019. *Physokermes*			
1. *piceae* (Schrank)	*Abies bornmülleriana, Picea pungens*	Palaearctic	Mar. R., C. A. R.
020. *Pulvinaria*			
1. *betulae* (Linnaeus)	*Ostrya carpinifolia, Pyrus communi, Vitis vinifera,*	Cosmopolit	C. A. R., Mar. R., A. R.

Table 1 (cont.)

	Host plants	Zoogeographical region	Distribution in Turkey
	Salix sp., *Vitis* sp., *Rosa* sp., *Prunus armeniaca*		
2. *vitis* Signoret	*Vitis vinifera*	Cosmopolit	C. A. R.
021. *Rhizopulvinaria*			
1. *artemisiae* (Signoret)	*Acantholimon echinus,* *Acathopyllum* sp., *Artemisia* sp., *Cerastium* sp., *Dianthus* sp., *Gypsophila* sp., *Kochia* sp. *Rosa* sp., *Silene* sp., *Scrophularia* sp., *Scutellaria* sp.	Palaearctic	C. A. R.
2. *halli* Borchsenius	–	Palaearctic	–
022. *Saissetia*			
1. *coffeae* (Walker)	Polyphag on fruits and ornamentals	Cosmopolit	B. R., C. A. R., Mar. R.
2. *oleae* (Olivier)	*Olea* spp., all fruit trees, ornamentals, *Cycas revoluta,* *Tamarix* sp.	Cosmopolit	Mar. R., A. R., Med. R.
3. *nigra* (Nietner)	*Myrtus communis*	Mediterranean	Med.R.
023. *Sphaerolecanium*			
1. *prunastri* (Fonscolombe)	Stone fruits	Nearctic, Palaearctic	C. A. R., Med. R., B. R.,A. R.
CRYPTOCOCCIDAE			
024. *Cryptococcus*			
1. *fagisuga* Lindinger	*Fagus orientalis*	Nearctic, Palaearctic	B. R.
025. *Pseudochermes*			
1. *fraxini* (Kaltenbach)	*Fraxinus excelsior*	Palaearctic	Mar. R.
DACTYLOPIIDAE			
026. *Dactylopius*			
1. *coccus* Costa	*Opuntia ficus*	Cosmopolit	Med. R.
DIASPIDIDAE			
027. *Acanthomytilus*			
1. *cedricola* (Balachowsky and Alkan)	*Cedrus* spp.	ENDEMIC	C. A. R., A. R., Mar. R., S. A. R.
028. *Adiscodiaspis*			
1. *tamaricicola* Malenotti	*Tamarix pallasii, T. pentandra*	Palaearctic	C. A. R., A. R.
029. *Aonidi*			
1. *lauri* (Bouché)	*Laurus nobilis*	Cosmopolit	Med. R., S. A. R., Mar. R.

Table 1 (cont.)

	Host plants	Zoogeographical region	Distribution in Turkey
2. *cupressaspis* (Lindinger)	*Cupressus sempervirens*	Palaearctic	Med. R.
030. *Aonidiella*			
1. *aurantii* (Maskell)	*Acacia* spp., *Citrus* spp., *Rosa* spp., *Amaranthus viridis,*	Cosmopolit	Med. R., A. R.
2. *citrina* (Coquillett)	*Acacia cultiformis, Catalpa bignonoides, Ceratonia siliqua, Citrus* spp., *Elaeagnus angustifolia, Hedera helix, Euonymus* spp., *Jasminum* spp., *Rosa* spp., *Vitis vinifera*	Cosmopolit	Med. R., A. R.
031. *Aspidiotus*			
1. *hedericola* (Leonardi)	*Laurus nobilis, Hedera helix*	Mediterrannean	Med. R., A. R., Mar. R.
2. *nerii* (Bouché)	*Acacia cultiformis, A. cyanophilla, Aucuba japonica, Asparagus aqutifolius, Campis radicans, Canna indica, Cedrus libani, Citrus limon, Cycas revaluata, Hedera helix, Jasminum* spp., *Laurus nobilis*	Cosmopolit	Med. R., A. R., Mar. R., B. R.
3. *spinosus* Comstock	*Polyphag, Viburnum tinus*	Palaearctic	Med. R.
032. *Aulacaspis*			
1. *rosae* (Bouché)	*Rosa* spp., *Rubus fruticasus*	Cosmopolit	Med. R., Mar. R., B. R.
033. *Carulaspis*			
1. *carueli* (Signoret)	*Arceuthos drupacea, Chammaecyparis lawsoniana, Cupressus arizonica, Juniperus communis, Thuja orientalis*	Cosmopolit	Med. R., A. R.
2. *juniperi* (Bouché)	*Cupressus sempervirens, T. occidentalis Juniperus exelsa, Thuja orientalis*	Cosmopolit	Med. R.
034. *Chionaspis*			
1. *etrusca* Leonardi	*Tamarix pallasii, Tamarix* spp.,	Cosmopolit	Med. R. C. A. R., B. R., A. R.
2. *salicis* (Linnaeus)	*Populus alba, P. canadensis-hybriden, P. nigra, P. tremulae, Salix alba, S. babylionica, Ulmus* spp.	Cosmopolit	Med. R. C. A. R., B. R., A. R. E. A. R. Mar. R.
035. *Chrysomphalus*			
1. *aonidum* (Linnaeus)	*Citrus limon, C. sinensis, Palmae* spp.	Cosmopolit	C. A. R., A. R., B. R.
2. *dictyospermi* (Morgan)	*Aloe* spp., *Aralia* spp., *Buxus microphylla, Citrus auratium Ceratonia siliqua, Citrus sinensis, Dracenea* spp., *V. amara, D. deremensis, Eribotrya japonica, Senecio bicolor*	Cosmopolit	Med. R., A. R., B. R.

Table 1 (cont.)

	Host plants	Zoogeographical region	Distribution in Turkey
3. *pinnulifer* (Maskell)	*Citrus auratium, C. bigaradia, C. limon, Euonymus japonica, Ficus carica, Taxus* spp.	Cosmopolit	Med. R., A. R., B. R.
036. *Contigaspis*			
1. *zillae* (Hall)	*Acontholium* spp.	Palaearctic	S. A. R.
037. *Diaspidiotus*			
1. *caucasicus* (Borchsenius)	*Populus nigra* v. *pyramidalis*	Palaearctic	C. A. R., B. R.
2. *distinctus* (Leonardi)	*Ephedra campyllopoda*	Palaearctic	Med. R.
3. *osborni* (Newell and Cockerell)	*Salix* spp.	Cosmopolit	E. A. R.
4. *prunorum* (Laing)	*Prunus domestica, P. dulcis*	Palaearctic	
5. *wuenni* (Lindinger)	*Alnus* spp.	Palaearctic	
038. *Diaspis*			
1. *bolsduvalii* Signoret	*Orchis* spp., *Palmae* spp.	Cosmopolit	C. A. R.
2. *bromelia* (Kerner)	*Orchis* spp.	Cosmopolit	C. A. R.
3. *echinocacti* (Bouché)	*Cactus* spp., *Opuntia ficus india*	Cosmopolit	C. A. R. Med. R.
4. *syriaca* (Lindinger)	*Pistacia terebinthus, P. vera*	Mediterrannean	Med. R.
039. *Duplachionaspis*			
1. *berlesii* (Leonardi)	*Artrocnemum gloucum*	Mediterranean	
2. *stanotophri* (Cooley)	*Phragmites australis*	Mediterranean	Med. R.
040. *Dynaspidiotus*			
1. *abieticola* (Koroneos)	*Abies bornmülleriana*	Mediterranean	C. A. R.
2. *abietis* (Schrank)	*Abies* spp., *Pinus* spp.	Palaearctic	C. A. R., B. R., Mar. R., A. R.
3. *atlanticus* (Balachowsky)	*Olea europea*	Mediterranean	Med. R.
4. *biritannicus* (Newstead)	*Ceratonia siliqua, Hedera helix, Laurus nobilis, Pistacia lentiscus*	Cosmopolit	Med. R., Mar. R., A. R.
041. *Epidiaspis*			
1. *gennadii* (Leonardi)	*Pistacia* spp.	Mediterranean	Med. R., B. R., S. A. R., C. A. R.
2. *leperii* (Signoret)	*Pistacia* spp., *Prunus* spp.	Nearctic, Palaearctic	Med. R., B. R., S. A. R., C. A. R.
042. *Fiorinia*			
1. *floriniae* (Targioni-Tozzetti)	*Livistonea chinensis, Palmae* sp. *Phoenix* spp., *Ruscus hypoglossum*	Cosmopolit	Mar. R.
043. *Furcadiaspis*			
1. *zaminae* (Morgan)	*Bricardia vinicera, Cycas revoluata, Palmae* spp.	Cosmopolit	Mar. R.

Table 1 (cont.)

	Host plants	Zoogeographical region	Distribution in Turkey
044. *Genistaspis*			
1. *zelihae* (Bodenheimer)	*Genista joubertii inops*	ENDEMIC	C. A. R.
045. *Gonaspidiotus*			
1. *minimus* (Leonardi)	*Quercus coccifera, Q. dschrochensis, Q. ilex*	Mediterranean	Med. R., Mar. R., A. R.
2. *seurati* (Marchal)	*Thuja* spp.	Mediterranean	
046. *Hemiberlesia*			
1. *latania* (Signoret)	*Prunus dulcis, Strelizia* spp.	Palaearctic	Mar. R., A. R.
2. *rapax* (Comstock)	*Euonymus japonicus*	Palaearctic	Mar. R. A. R.
3. *cyanophylii* (Signoret)	*Brasiliopuntia brasiliensis, Chamacerasus silvestri, Cactus* spp., *Dianthus caryophyllus, Senecio bicolor Gasteria maculata, G. verricosa,*	Cosmopolit	C. A. R., Mar. R., A. R.
047. *Kuwanaspis*			
1. *pseudoleucaspis* (Kuwana)	*Bambusa* spp.	Cosmopolit	Mar. R.
048. *Lepidosaphes*			
1. *beckii* (Newman)	*Citrus* spp., *Malus sylvestris*	Palaearctic	Med. R., C. A. R., Mar. R.
2. *conchiformis* (Gmelin)	*Ficus carica, Labiata* spp., *Rhamnus* spp., *Ulmus* spp.	Cosmopolit	Med. R., A. R.
3. *gloverii* (Packard)	*Citrus auratium, C. limon, C. sinensis*	Palaearctic	Med. R., A. R., B. R.
4. *granati* Koroneos	*Ficus caria, Acacia cultriform, Ulmus* sp., *Celtis* sp.	Palaearctic	Med. R.
5. *juniperi* Lindinger	*Cedrus libani, Pinus nigra, Thuja occidentalis*	Palaearctic	B. R., C. A. R., Mar. R., A. R.
6. *malicola* Borchsenius	*Prunus* sp.	Palaearctic	C. A. R.
7. *newsteadi* (Sulc)	*Abies bornmülleriana, A. pinsapa, Picea pungens*	Mediterrannean, Palaearctic	C. A. R., Mar. R.
8. *pinnaeformis* (Bouché)	Polyphag	Cosmopolit	E. A. R., Med. R.
9. *pistaciae* Archangelskaia	*Malus sylvestris, Pistacia lentiscus, P. tenebinthus*	Palaearctic	A. R., B. R.
10. *pistacicola* Borchsenius	*Pistacia vera*	Palaearctic	S. A. R.
11. *ulmi* (Linnaeus)	*Acer negunda, Bauhinia* spp., *Betula* spp., *Ligustrum* spp., *Malus sylvestris, Salix babylanica, Pistacia lentiscus, Tamarix* spp., *Vinca major*	Cosmopolit	Med. R., C. A. R., B. R., A. R. E. A. R.
12. *serrifrons* (Leonardi)	*Pinus brutia, Thuja occidentalis*	ENDEMIC	A. R.
049. *Leucaspis*			
1. *knemion* Hoké	*Pinus* sp.	Mediterrannean	S. A. R.

Table 1 (cont.)

	Host plants	Zoogeographical region	Distribution in Turkey
2. *loewi* Colvée	*Pinus* spp.	Palaearctic	Med. R., C. A. R., A. R., B. R.
3. *pini* (Hartig)	*Olea europea, Pinus pinea*	Palaearctic	Med. R., Mar. R.
4. *pusilla* Löw	*Cedrus* spp., *Pinus* spp.	Palaearctic	Mar. R., Med. R., A. R.,C. A. R.
5. *riccae* Targioni-Tozzetti	*Ephedra* spp., *Euphorbia* spp., *Olea europea*	Palaearctic	Med. R., S. A. R.
050. *Lineaspis*			
1. *striata* (Newstead)	*Thuja occidentalis, Cupressus sempervirens, Cupressus* sp., *Arceuthobium* spp., *Thuja* spp.	Palaearctic	Med. R.
051. *Lopholeucaspis*			
1. *japonica* (Cockerell)	Polyphag, *Citrus* spp.	Cosmopolit	B. R.
052. *Melanaspis*			
1. *inopinata* (Leonardi)	*Arbudus unedo, Bauhinia* spp., *Cercis siliquastrum, Prunus avium*	Palaearctic	E. R., C. A. R., Med. R. E. R.
053. *Mercetaspis*			
1. *halli* (Green)	*Astragallus* spp., *Prunus armenica*	Cosmopolit	Med. R., S. A. R., C. A. R., E. R.
2. *sureyanus* (Bodenheimer)	*Astragallus* spp.	Palaearctic	C. A. R.
054. *Mohelnaspis*			
1. *massiliensis* (Goux)	*Alopecurus myosuorides* v. *myosuorides, A. agrestis*	Palaearctic	C. A. R.
055. *Neochionaspis*			
1. *asiatica* (Archangelskaia)	*Prunus communis*	Palaearctic	Med. R.
056. *Parlatoria*			
1. *crotonis* Douglas	*Citrus* sp.,	Cosmopolit	Med. R., E. R., C. A. R.
2. *oleae* (Colvée)	Polyphag, *Eriabortia* spp., *Fraxinus* spp., *Rosea* sp. *Malus sylvestris, Prunus* spp., *Syringa vulgaris,*	Cosmopolit	Turkey
3. *pergandi* Comstock	*Citrus* spp., *Malus sylvestris*	Cosmopolit	Med. R., E. R., Mar. R.
4. *ziziphi* (Lucas)	*Asparagus* spp., *Citrus* spp.	Cosmopolit	Med. R., E. R.
5. *parlatoriae* (Sulc)	*Abies bormmülleriana*	Palaearctic	B. R., C. A. R.
057. *Parlatoreopsis*			
1. *longispinus* Newstead	*Acacia cultriformis, Acer rubrum, Orchis* spp., *Celtis* spp.	Palaearctic	Med. R.
058. *Pinnaspis*			
1. *aspidistrae* (Signoret)	*Asplenium* spp., *Aspidistra elatiorum*	Cosmopolit	Mar. R.
059. *Pseudaulacaspis*			
1. *pentagona* (Targioni-Tozzetti)	Polyhpag on fruits and ornamentals	Palaearctic	Med. R., C. A. R. , B. R., E. A. R.

Table 1 (cont.)

	Host plants	Zoogeographical region	Distribution in Turkey
060. *Quadraspidiotus* (Diaspidiotus)			
1. *anatolica* (Bodenheimer)	*Prunus dulcis, Amygdalus* spp.	ENDEMIC	C. A. R.
2. *armenicus* (Borchsenius)	*Populus nigra, Salix* spp.	Palaearctic	E. A. R.
3. *gigas* (Thiem and Gerneck)	*Populus* spp.	Cosmopolit	E. R., Mar. R., E. A. R.
4. *lenticularis* (Lindinger)	*Prunus avium*	Cosmopolit	Mar. R.
5. *marani* (Zahradnik)	*Fraxinus* spp., *Malus slyvestris, Platanus orientalis*	Palaearctic	Med. R., C. A. R., B. R., E. A. R.
6. *ostreaeformis* (Curtis)	*Populus nigra, Salix* spp.	Cosmopolit	B. R., A. R., Mar. R.
7. *perniciosus* (Comstock)	Polyphag on fruits and ornamentals	Cosmopolit	Med. R., C. A. R., B. R., E. A. R.
8. *pyri* (Lichtenstein)	*Malus slyvestris, Salix* spp.	Cosmopolit	C. A. R., A. R., Mar. R., E. A. R.
9. *zonatus* (Fauenfeld)	*Fagus orientalis, Salix* spp., *Ulmus americana*	Palaearctic	B. R., C. A. R., Mar. R., E. A. R.
061. *Rhizaspidiotus*			
1. *canariensis* (Lindinger)	*Artemisia fragrans*	Palaearctic	C. A. R.
2. *donacis* (Leonardi)	*Phragmites australis*	Palaearctic	Med. R.
062. *Salicicola*			
1. *archangelskyae* (Archangelskaia)	*Olea europea, Prunus avium*	Palaearctic	B. R., C. A. R., Med. R.
2. *kermanensis* (Lindinger)	*Salix alba, Populus* spp.,	Palaearctic	Med. R.
3. *pistaciae* (Lindinger)	*P. lentiscus, Pistacia* spp.	Palaearctic	Med. R., S. A. R., A. R.
063. *Targionia*			
1. *nigra* Signoret	*Gleditschia* sp.	Palaearctic	Med. R.
2. *vitis* (Signoret)	*Castanea crenata, Vitis vinifera, Quercus* sp.	Palaearctic	C. A. R., Med. R., Mar. R.
064. *Unaspis*			
1. *eunoymi* (Comstock)	*Buxus sempervirens, Rosa* spp., *Eunoymus orgentata*	Cosmopolit	B. R., C. A. R., Mar. R., A. R.
ERIOCOCCIDAE			
065. *Eriococcus*			
1. *buxi* (Fonscolombe)	*Buxus sempervirens*	Palaearctic	B. R.
066. *Gossyparia*			
1. *spurius* (Modeer)	*Ulmus* spp.	Nearctic, Neotropic, Palaearctic	Med. R., C. A. R.
067. *Greenisca*			
1. *laingi* (Bodenheimer)	–	Palaearctic	Mar. R.

Table 1 (cont.)

	Host plants	Zoogeographical region	Distribution in Turkey
068. *Pseudochermes*			
1. *fraxini* Kaltenbach	–	Palaearctic	–
069. *Rhizococcus*			
1. *araucariae* Maskell	*Araucaria* sp.	Cosmopolit	Mar. R.
KERMESIDAE			
070. *Kermes*			
1. *bekiri* Bodenheimer	*Quercus aegilops*	ENDEMIC	A. R.
2. *muhlisi* Bodenheimer	*Quercus* sp.	ENDEMIC	Med. R.
3. *roboris* (Fourcroy)	*Qercus* sp.	Palaearctic	
4. *sadrii* Bodenheimer	*Quercus aegilops*	ENDEMIC	E. A. R.
5. *safinazae* Özkök	*Q. cerris, Q. dscorochensis, Qercus* sp.	Mediterrannean	Med. R.,
6. *vermilio* (Planchon)	*Qercus coccifera, Q. ilex, Q. suber*	Mediterrannean	A. R.
071. *Nidularia*			
1. *balachowskyi* Bodenheimer	*Quercus* sp.	Palaearctic	S. A. R.
2. *pulvinata* (Planchon)	*Q. coccifera, Q. ithaburensis, Quercus* sp.	Palaearctic	
LECANODIASPIDIDAE			
072. *Lecanodiaspis*			
1. *sardoa* Targioni-Tozzetti	*Cistus albida*	Palaearctic	A. R.
073. *Gueriniella*			
1. *serratulae* (Fabricius)	Polyphag on some fruits, and Compositae	Palaearctic	Med. R., C. A. R., Mar. R., A. R.
MARGARODIDAE			
074. *Icery*			
1. *purchasi*	Polyphag on citrus trees, and ornamentals	Cosmopolit	Med. R., Mar. R., B. R., A. R.
075. *Marchalina*			
1. *hellenica* (Gennadius)	*Pinus brutia, P. halapensis, P. pinea, P. silvestris*	Mediterrannean, Australia	Med. R., A. R., Mar. R.
076. *Palaeococcus*			
1. *fuscipennis* Burmeister	–	Palaearctic	–
077. *Porphyrophora*			
1. *hamelii* Brandt		Palaearctic	E. A. R.

Table 1 (cont.)

	Host plants	Zoogeographical region	Distribution in Turkey
2. *tritici* (Bodenheimer)	*Gramineae*	Palaearctic	C. A. R., S. A. R.
MICROCOCCIDAE			
078. *Micrococcus*			
1. *similis* Leonardi	_	Mediterrannean	Mar. R.
ORTHEZIIDAE			
079. *Orthezia*			
1. *urticae* (Linnaeus)	Polphag on various herbaceous	Cosmopolit	Med. R., C. A. R., Mar. R.
PSEUDOCOCCIDAE			
080. *Chaetococcus*			
1. *bambusae* (Maskell)	*Bambusa* sp.	Cosmopolit	B. R.
081. *Dysmicoccus*			
1. *multivorus* (Kiritchenko)	*Eryngium* sp., *Malva* sp., *Medicago sativa*, *Bunium* sp., *Nepeta* sp., *Dacus guttatus*,	Palaearctic	Med. R.
082. *Metadenopus*			
1. *ankaranus* (Bodenheimer)	*Festuca ovina*	ENDEMIC	C. A. R.
083. *Mirococcus*			
1. *inermisn* (Hall)	*Chenopodium album*, *Heliotrophium earopeum*, *Polygonum* sp., *Salsola kali*	Palaearctic, Ethiopian	E. A. R.
084. *Nipaecoccus*			
1. *filamentosus* (Cockerell)	*Robinia pseudacacia*	Nearctic, Neotropic, Palaearctic	Mar. R.
2. *nipae* (Maskell)	Polyphag on fruit trees	Cosmopolit	Mar. R.
085. *Peliococcus*			
1. *chersonensis* (Kiritchenko)	*Artemisia fragrans*, *Cnopis* sp., *Globularia* sp., *Solanum tuberosum*	Palaearctic	C. A. R., E. A. R.
086. *Phenacoccus*			
1. *aceris* (Signoret)	Polyphag on Acer sp., *Platanus* sp. and fruit trees	Nearctic, Neotropic, Palaearctic	C. A. R.
2. *avenae* (Bouche)	Ornamentals	Palaearctic	C. A. R.
3. *asphodeli* (Goux)	*Asphodelus microcarpus*	Palaearctic	C. A. R.
4. *graminicola* Lindinger	*Dactylis* sp., *Melilotus* sp., *Hordeum vulgare*	Palaearctic	A. R.

Table 1 (cont.)

	Host plants	Zoogeographical region	Distribution in Turkey
087. *Planococcus*			
1. *citri* (Risso)	Polyphag on citrus trees, and ornamentals	Cosmopolit	Med. R., Mar. R., A. R., C. A. R.
2. *ficus* (Signoret)	*Ficus* sp., *Vitis* sp., *Punica granatum*	Cosmopolit	A. R., C. A. R., Mar. R.
3. *vovae* (Nasonov)	*Cupressus sempirvirens, C. goveniana, Cupressus* sp., *Juniperis excelsa, Laurus nobilis, Libocedrus decurrens, Taxus baccata, Thuja occidentalis*	Cosmopolit	Med. R., C. A. R., Mar. R.
088. *Pseudococcus*			
1. *caricus* (Gennadius)	*Pinus laricio*	ENDEMIC	–
2. *laingi* Bodenheimer	–	ENDEMIC	C. A. R.
3. *longispinus* (Targioni-Tozzetti)	*Acer* sp., *Ficus carica, Hedera helix, Nerium oleander, Robinia pseudacacia*	Nearctic, Neotropic, Palaearctic	Med. R., Mar. R., A. R.
4. *viburni* (Signoret)	Polyphag on forest trees, *Citrus* spp.	Australian, Nearctic,	C. A. R.,Mar. R., B. R., Med. R., A. R.
089. *Trabutina*			
1. *mannipara* Hemprich and Ehrenberg	*Tamarix* sp.	Palaearctic	
2. *crassispinosa* Borchsenius	*Tamarix* sp.	Palaearctic	Med. R.
090. *Trionymus*			
1. *cressae* (Hall)	*Euphorbia* sp.	Palaearctic	C. A. R.
2. *graminellus* (Borchsenius)	*Caucalis* (cf) *ptatycorpis*	Palaearctic	E. A. R.

snowy winters and dry summers. They get rain mainly in spring time. The northern parts Marmara and Blacksea are cooler than southern regions and they have snow during the winters.

The present list of scale insects from Turkey is based on collecting records of the authors and on bibliographic sources. In this list the known host plant, zoogeographical region and distribution in Turkey according to geographical regions were given. The 176 species on the list belong to 13 families, 84 genera. Including in the list 12 species are considered to be endemic; Coccidae: *Nemolecnium aptii* (Bodenheimer), *Parthenolecanium tamarics* (Bodenheimer); Diaspididae: *Acanthomytilus cediricola* (Balachowsky and Alkan), *Genistaspis zelihae* (Bodenheimer), *Lepidosaphes serrifrons* (Leonardi), *Diaspidiotus anatolica* (Bodenheimer); Kermesidae: *Kermes bekiri* Bodenheimer, *K. muhlisi* Bodenheimer, *K. sadrii* Bodenheimer; Pseudococcidae: *Metadenopus ankaranus* (Bodenheimer), *Pseudococcus caricus* (Gennadius), *P. laingi* Bodenheimer. These have mostly recorded from Central Anatolia which has rather little geographical interaction with other parts of Turkey. Many of these endemic species belong to little known groups such as the Kermesidae, which has six species in Turkey, three of endemic. In addition, a total of 10 Diaspididae (Erler et al., 1996; Uygun et al., 1998) and 2 Pseudococcidae (Uygun et al., 1998) not previously recorded from Turkey have been found in recent years, suggesting that the scale insect fauna of Turkey is still rich in undetermined species.

The most numerous families are the Diaspididae (93 species), Coccidae (33 species) and Pseudococcidae (22 species). The Asterolecaniidae, Cerococcidae, Cryptococcidae, Dactylopiidae, Eriococcidae, Kermesidae, Lecanodiaspididae, Margarodidae, Micrococcidae and Ortheziidae are presented with low number of species from 1 to 6 species.

The species list also shows the zoogeographical region, distribution in Turkey on geographical basis, known hosts in Turkey and literature sources. Among the 173 species, some are widely distributed in Turkey and some may have been introduced. Of 22 species on agricultural crops, 15 require periodical monitoring and either chemical or biological control when necessary. These species are: Diaspididae: *Diaspidiotus* (Quadraspidiotus) *perniciosus* Comstock, *Epidiaspis leperii* (Signoret), *Pseudaulacaspis pentagona* (Targioni-Tozzetti), *Aonidiella aurantii* (Maskell), *Lepidosaphes ulmi* (Linnaeus), *Parlatoria oleae* (Colveé), *Mercetaspis halli* (Green), *Salicicola pistaciae* (Lindinger); Coccidae: *Sphaerolecanium prunastri* (Fonscolombe), *Palaolecanium bituberculatum* (Signoret), *Parthenolecanium corni* (Bouché), *Chloropulvinaria floccifera* (Westwood), *Ceroplastes floridensis* Comstock; Margarodidae: *Icerya purchasi* Maskell and Pseudococcidae: *Planococcus citri* Risso.

A characteristic of the countryside of Turkey is the great range of natural environments and this is reflected by the scale insect species recorded, which include species associated with the Euro-Siberian, Mediterranean and Irano-Turanian (Kozar, 1995). The Mediterranean species are mainly found in Mediterranean, Aegean and Marmara regions. The geographical position of Turkey on the Eastern edge of Asia has meant that there has been a great movement of plant material through it, thus leading to the introduction of many exotic species. Furthermore, the climate allows the establishment of tropical and subtropical species.

This list is considered to provide a starting point for further investigations. Despite the increase in faunistic research in the last few years, the scale insect fauna of Turkey is still very incompletely known and, in particular further studies on the Cerococcidae, Eriococcidae, Kermesidae, Margarodidae, Ortheziidae and Pseudococcidae are needed.

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Ants (Hymenoptera:Formicidae) Associated with Soft Scale Insects in Turkey: A Preliminary List

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Ten ant species were found associated with 12 coccid species on various host plants in Turkey. These are *Formica cunicularia* Latreille, *Camponatus aethiops* (Latreille), *C. sanctus* Forel, *Crematogaster ionia* Emery, *Dolichoderus quadripunctatus* (L.), *Lasius alineus* (Foerster), *Plagiolepis vindobonensis* Lomnicki, *Prenolepis nitens* (Mayr), *Tapinoma erraticum* (Latreille) and *Tetramorium ferox* Ruzsky, (Hymenoptera: Formicidae). In the study new ant-scale insect association were observed. The coccid species visited by these ants, their host plants and localities are also presented.

Keywords: soft scale insects, Formicidae.

Many honeydew producers, like soft scale insects, are often found in association with other insects, particularly ants, wasps and bees. From these associations, ants and coccids is a well-known phenomenon (Bodenheimer, 1951; Schmutterer, 1952; Way, 1963; Rosen, 1967; Hamon and Williams, 1984; Kosztarab and Kozár, 1988; Bach, 1991; Buckley and Gullan, 1991; Gullan, 1997). These associations are usually beneficial to both soft scale and associate ants. Benefits of coccids to ants are in a way that the coccids serve as a source of proteins and lipids in addition to carbohydrate from the honeydew (Evans and Leston, 1971; Ho and Khoo, 1997). Benefits of ants to coccids are removal of honeydew which provides sanitation of soft scales aggregations by reducing physical fouling caused by both the sugary excreta and the sooty molds that grow on it (Balakrishnan et al., 1992; Gullan, 1997). There is unequivocal evidence that ants can protect scale insects from their natural enemies (Bartlett, 1961; Hanks and Sadof, 1990; Buckley and Gullan, 1991; Bach, 1991). Coccids mortality from parasitism and other reasons decreased in presence of ants (Bartlett, 1961; Bradley, 1973; Bach, 1991; Reimer et al., 1993). For this reason, the coccids are densely populated in presence of ants (Bach, 1991; Sugonyaev, 1995).

On the other hand, there is some evidence that presence of ants has no effect on mortality from parasitism and predatory (Rosen, 1967; Itioka and Inoue, 1996).

Certain ant species are known to transport coccids to new feeding sites on the same plant or to uninfested plants, thus greatly facilitating the spread of soft scale populations (Way, 1963). Some species of ants build cartons or tents over colonies of soft scale insects. These ant tents provide some protection from weather, but more importantly, shields the colony from attack by parasites and predators (Hamon and Williams, 1984; Sugonyaev, 1995). A few taxa of tropical or subtropical scale insects which live in ant nests or shelters, display obvious behavioural and morphological adaptations (Hodgson, 1994;

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Gullan, 1997). Some predatory ants that are dependent on honeydew, such as *Oecophylla* spp., *Dolichoderus thoracicus* and *Azteca* spp., may be used in the biological control of a range of artropods (Way and Khoo, 1992).

The association of ants and homopters which is a very common phenomen in plants have not been extensively studied in Turkey. The only two studies concerning this subject are by Elmalı (1993) and Ülgentürk and Toros (1998). Therefore, the data is scarce. The present study aims to underline the association of ants with coccid species in the selected areas. The host plants, where this association is observed, were determined.

Methods

The coccids infesting plants, particularly woody ornamental and fruit plants in Turkey, were studied in a series of Aegean (Balıkesir, Denizli, Muğla), Central Anatolian (Afyon, Ankara, Konya, Niğde, Sivas) and Mediterranian (Burdur, Isparta) regions in unperiodical surveys between 1995–1998. During these surveys, a special care was given to collect the samples of the ants attending the colonies of coccids, regularly in many parks and fruit groves. The ants and coccids then, were examined and identified.

Results and Discussion

Colonies of soft scale insects, Coccus sp., *Coccus hesperidum* L., *Eulecanium ciliatum* (Douglas), *Eulecanium tiliae* (Linnaeus), *Filippia follicularis* (Targioni-Tozzetti), *Parthenolecanium* sp., *Parthenolecanium corni* (Bouché), *Physokermes piceae* (Schrank), *Rhizopulvinaria* sp., *Rhodococcus* sp., Saissetia coffeae (Walker), and *Sphaerolecanium prunastri* (Fonscolombe) were frequently found to be under regular ant attendance. The ants involved are listed *(Table 1).*

Camponatus aethiops (Latreille) and *Dolichoderus quadripunctatus* (L.) visited together six coccids species; *C. hesperidum, E. ciliatum, E. tiliae, Parthenolecanium* sp., *P. corni* and *S. coffeae* in different localities. On the other hand, *Tapinoma erraticum* (Latreille) visited only *F. follicularis* on *Fraxinus* in Ankara. *S. prunastri* were associated with different ant species which are *C. aethiops, D. quadripunctatus, Lasius alineus* (Foerster), *Prenolepis nitens* (Mayr) and *Tetramorium ferox* Ruzsky. All these ant species were found on bark, branches and leaves with coccid species but *Camponotus sanctus* Forel is found on roots of *Acantholimon* sp. which is assotiated with *Rhizopulvinaria* sp. Some of the ants were also observed in association with different coccids on various plants *(Table 1).* For example, *C. aethiops* got some honeydew by visiting the *C. hesperidum* on *Nerium oleander, E. ciliatum* on *Acer* sp. and *S. prunastri* on *Prunus* sp.

During the study, it was determined that the species listed above visited young females and females in preoviposition especially during May and mid-June. This was because of the fact that the secretion of honeydew reached its maximum during this period. The amount of honeydew produced by larvae that appears during June to Sep-

tember increased with the increase of population, although it was not high compared to young females. It was also found that the first and second instar larvae of coccids living on branches of leaves were visited by ants.

Table 1

Ant species visiting various coccids on different host plant in various provinces in Turkey

Ants	Coccids	Host plant of coccids	Localities
Camponatus aethiops (Latreille)	*Eulecanium ciliatum*	*Acer pseudoplatanus*	Ankara
„	*E. ciliatum*	*Prunus domestica*	Niğde
„	*Eulecanium tiliae*	*Craetagus* sp.	Sivas
„	*Parthenolecanium corni*	*Malus communis*	Ankara
„	*Sphaerolecanium prunastri*	*Prunus* sp.	Isparta
„	*Coccus hesperidum*	*Nerium oleander*	Ankara
Camponatus sanctus Forel	*Rhizopulvinaria* sp.	*Acantholimon* sp.	Ankara
Cremastogaster ionia Emergy	*Coccus* sp.	*Prunus cerasifera*	Burdur
Dolichoderus quadripunctatus (L.)	*S. prunastri*	*Prunus amygdalus*	Isparta
„		„	Afyon
„	*Saissetia coffeae*	*Pyracantha coccinea*	Balıkesir
„	*P. corni*	„	Balıkesir
„	*Parthenolecanium* sp.	*Juglans regia*	Burdur
Formica cunicularia Latreille	*E. ciliatum*	*Prunus persica rosa plena*	Ankara
„	„	*Acer campestre*	Ankara
„	„	*Acer pseudoplatanus*	Ankara
„	*Physokermes piceae*	*Piceae pungens*	Ankara
Lasius alineus (Foerster)	*E. ciliatum*	*Acer campestre*	Ankara
„	„	*Prunus cerasus*	Ankara
„	*S. prunastri*	*Prunus domestica*	Isparta
Plagiolepis vindobonensis	*E. ciliatum*	*Crateagus oxycantha*	Ankara
„	*E. tiliae*	„	Ankara
Prenolepis nitens (Mayr)	„	„	Isparta
„	„	*Prunus domestica*	Konya
Tapinoma erraticum (Latreille)	*Filippia follicularis*	*Fraxinus* sp.	Ankara
Tetramorium ferox Ruzsky	*S. prunastri*	*Prunus domestica*	Ankara
„	*Rhodococcus* sp.	*Rosa canina*	Sivas

Records of *Camponatus sanctus, Prenolepis nitens, Tapinoma erraticum* and *Tetramorium ferox* has not been found in literature. These four species are new records for associated ant species with coccoids.

L. alineus visited *E. ciliatum* and *S. prunastri* in Turkey and it was found associated with *Chanaurococcus parvus* (Borchsenius), *Chanaurococcus subterraneus* (Newstead), *Coccura comari* (Künow), *Trionymus elymi* (Borchsenius) and *Trionymus multivorus*

(Kiritchenko) (Pseudococcidae; Homoptera) in Oriental Kazakhstan and Polond (Kosztarab and Kozár, 1988). *E. ciliatum* and *S. prunastri* are new records for *L. alineus.* This ant species was also found associated with two aphid species living on the roots of wheat namely with *Forda marginata* Koch (Hom., Pemphigidae) and *Anoecia* sp. (Homoptera: Thelaxidae) on roots of wheat in Konya, Turkey (Elmalı, 1993).

In addition, *Dolichoderus quadripunctatus* was recorded associated with *P. corni* (Schmutterer, 1952), but in our work *D. quadripunctatus* visited *S. coffeae, S. prunastri* and *P. corni* in Turkey.

Visits of *Camponatus aethiops* (Latreille), *Formica cunicularia* Latreille and *Plagiolepis vindobonensis* Lomnicki (Hymenoptera: Formicidae) to *Eulecanium ciliatum* (Douglas) on *Acer* spp. and *Crateagus* spp. was reported earlier in Turkey (Ülgentürk and Toros, 1998), but visits of *C. aethiops* to *Eulecanium tiliae, P. corni, S. prunastri* and *C. hesperidum; F. cunicularia* to *P. piceae* and *P. vindobonensis* to *E. tiliae* are have been newly observed in our work.

Associated ant species with scale insects besides our findings are *Anonychomyrma* sp. *Anoplolepis* sp., *Azteca* sp., *Cardiocondyla* sp., *Cladomyrma* sp., *Iridomyrmex* sp., *Linepithema* sp., *Monomomorium* sp., *Nylanderia* sp., *Oecophylla* sp., *Papyrius* sp., *Pheidole* sp., *Philidris* sp., *Podomyrma* sp., *Pseudomyrmex* sp., *Solenopsis* sp., *Technomyrmax* sp., *Tetraponera* sp. and *Zacryptocerus* sp. all the world (Bodenheimer, 1951; Schmuterer, 1952; Bartlett, 1961; Way, 1963; Rosen, 1967; Bradley, 1973; Kosztarab and Kozár, 1988; Bach, 1991; Buckley and Gullan, 1991; Way and Khoo, 1992; Gullan, 1997).

In Israel, it was reported that *Plagiolepis ancyrensis* Santschi, *Tapinoma israelis* Forel, *Crematogaster jehovae* Forel, *Camponotus fellah* Emery had a common association with *C. hesperidum* and *Planococcus citri* on citrus (Rosen, 1967).

In this paper, only the association of ants with the coccids was presented. Their beneficial and harmful effects should be studied more extensively in order to use them in integrated pest management.

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Biology of *Euseius yousefi* (Acari: Phytoseiidae) Life Tables and Feeding Behaviour on Different Diets

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Effects on development, survivorship and reproduction of *Euseius yousefi* Zaher and El-Borolossy were studied in the laboratory using four different diets: the tetranychid mite *Tetranychus urticae* koch, the eriophyid mite *Eriophyes dioscoridis* Soliman and Abou-Awad, date palm pollen *Phoenix dactylifera* (L.) and sweet corn *Zea mays* L. pollen. The total development time ranged between 6.25 and 7.27 days for the adult female on animal diets and date palm pollen. In contrast, sweet corn pollen prolonged the developmental time and a higher nymphal mortality occurred. A total of 63.8, 50.5 and 57.6 eggs per female were obtained when eriophyid and tetranychid mites as well as date palm pollen, were provided, respectively. A diet of *T. urticae* provided the shortest generation time (T) and female longevity as well as the highest intrinsic rate of natural increase (rm = 0.294). A diet of *E. dioscoridis* resulted greatest female longevity and mean total fecundity which resulted in the highest net reproductive rate (Ro) value, intrinsic rate of increase (rm = 0.231) and finite rate of increase ($e^r m$ = 1.260) per day for *E. yousefi*. A diet of date palm pollen showed a close values of (T), (rm), ($e^r m$) and total eggs per female with a diet of eriophyid mite. The sex ratio of *E. yousefi* was strongly female biased (♀/total ranged 0.73–0.82). Sweet corn pollen was the least favorable food source, with shortest adult longevity and without reproductive activity.

Keywords: Acari, Phytoseiidae Pollenophagous, *E. yousefi*.

Phytoseiid mites have been characterized by their higher potenial rate of natural increase compared with their prey, Tetranychidae (Takafuji and Chant, 1976; Saito and Mori, 1981). Species of the genus *Euseius* have been classified as "pollenophagous", facilitative, general predator (Muma, 1971). Generalist phytoseiid species are common in subtropical tree crops and maintain themselves on pollen, fungi, and leaf sap when prey are scarce (McMurtry and Scriven, 1965; Grafton-Cardwell and Ouyang, 1996). Some phytoseiid mites reproduce more rapidly on pollen than on tetranychid mites (McMurtry and Scriven, 1964; Abou-Setta and Childers, 1987).

Euseius yousefi Zaher and El-Borolossy, a native phytoseiid mite, has been recorded from the New Vally (Egypt) on *Zizyptus spina – Christi,* L. – in association with eriophyid mite. A classification system of life style or predation types for phytoseiid mites has been proposed by (McMurtry and Croft, 1997). *A. yousefi* has been rated a type IV which is a generalist feeder on mites, but a specialist feeder on pollen. With the exception of the report by Momen (1999b), nothing is known about the development and food habits of this species, the present work deals with the biology and life table parameters of *E. yousefi* when provided with each of the following food sources: the tetranychid mite *Tetranychus urticae* koch, the eriophyid mite *Eriophyes dioscoridis* Soliman and Abou-Awad, and pollens of date palm *Phoenix dactylifera* (L.) and sweet corn *Zea mays* L.

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Materials and Methods

Maintenance of mite stock cultures

The laboratory stock culture of *E. yousefi* used in the present study originated from a *Zizyptus spina-Christi,* L. planted in New Vally, and reared on pollen from date palm *P. dactylifera* at 27 °C and 70–75% R. H.

Diets

Four diets were evaluated for their effect on development, survival, oviposition and life table parameter. Two broad categories of food were provided:

1. Animal:

a) *T. urticae* obtained from a laboratory colony reared under the same conditions as the predator. *T. urticae* was presented to female *A. yousefi* as immature stages (nymphs).

b) *E. dioscoridis* obtained from infested galls of the plant *Pluchea dioscoridis* (L.).

2. Pollen:

Two kinds of pollen were tested for their acceptability to *A. yousefi.*

a) Date palm *(P. dactylifera)* pollen

b) Sweet corn *(Z. mays)* pollen

Effect of diet on development and survival

The arenas used in this study were fabricated from (3 x 3 cm) of raspberry leaf discs placed with upper surface down on a water-saturated spong pad. A strip of moistened absorbent cotton was placed around the out side edge of the leaves. Single newly deposited eggs were placed on individual arenas supplied with the food resource to be evaluated. Arenas were examined daily and predator development and survival was recorded. Freshly collected pollen was made available to the predator by providing each arena with a small amount of test pollen and were replaced at 48–72 intervals. The pollen must store well and preferably have a low water content to minimize molding when used as a food source for mite rearing. Prey tetranychid and eriophyid mites consumed were replaced daily by fresh ones.

Effect of diet on oviposition, longevity and life table parameters

Newly emerged female, mated, confined individually on test arenas, along with food to be tested – A piece of leaf (¼ x ¼ cm) placed on the big veins of each leaf (arena), provided the mites with a shelter for resting and egg-laying and reduced their tendency to leave the leaf. Oviposition and survival were recorded all over female longevity. Phytoseiid eggs were removed daily for sex determination.

Every experiment was repeated twice and all were conducted under laboratory conditions at 27 + 1 °C and 70–80% R. H-life table parameters as defined by Birch (1948) were calculated according to a BASIC computer program (Abou-Setta et al., 1987).

Results

Behavior and feeding observation

The larval stage feed on pollen or animal diets. *E. yousefi* was able to develop to maturity and reproduce, respectively, utilizing a wide range of food substances under laboratory conditions. The colour of this fast moving phytoseiid mite from either field samples or pollen or eriophyid fed cultures was waxy yellow. Individual fed on *T. urticae* varied in colour from yellow to brown. Cannibalism was observed when food was scarce. Mating was essential for egg laying and occurred as early as the first few hours after maturity. Females mated more than once. Multiple matings were necessary for females to lay their full complement of eggs.

Effect of diet on development and survival

Successful development from egg to adult in *A. yousefi* occurred when the eriophyid, tetranychid and pollen of date palm or sweet corn were supplied as food *(Table 1)*. Mortality was high with *Z. mays* pollen and adult female were produced after 13.25 days, respectively. Mortality occurred during the protonymphal stage and when deutonymphal were moulting to adults. The developmental period was significantly rapid on animal diets and *P. dactylifera* pollen than on *Z. mays* pollen.

Effect of diet on oviposition, Longevity and life table

The preoviposition period likewise was similar on animal and pollen of date palm diets *(Table 1)*. Adult females of *A. yousefi* failed to lay any eggs when fed on *Z. mays* pollen and female longevity was extremely short *(Table 1)*. Female of *E. yousefi* fed on *E. dioscoridis* and pollen *P. dactylifera* had a significantly longer oviposition period and a greater total fecundity than female fed on *T. urticae nymphs (Tables 1 and 2)*.

Mean total fecundity was 63.80 and 57.60 eggs/♀ over a mean oviposition period of 37.67 and 38.27 days for female *E. yousefi* reared on *E. dioscoridis* and pollen of date palm compared to 50.5 eggs/♀ over a mean oviposition period of 20.68 days for predator female reared on *T. urticae*. Longevity of *E. yousefi* was significantly different when reared and maintained on *Z. mays* pollen ($x^- = 3.25$ days) compared to $x^- = 43.33$ days) when reared on *P. dactylifera* pollen *(Table 1)*.

Life table parameter

The mean generation time (T) of. *E. yousefi* fed *T. urticae* was shorter than that in case of *E. dioscoridis* and *P. dactylifera* diets. The higher net reproductive rate (Ro) value of 47.46 expected females per female was obtained with a diet of *E. dioscoridis (Table 2)*. The intrinsic rate of natural increase (rm) and, subsequently the finite rate of increase (erm) were relatively higher (0.2948 and 1.3429) when individuals were fed *T. urticae* compared with individuals fed *E. dioscoridis* and *P. dactylifera* pollen.

Table 1

Comparative duration (x ± S. E. in days) of female stage of *E. yousefi* at 27 °C on test food substrates

Developmental stages	*E. dioscoridis*	*T. urticae* (Nymph)	*P. dactylifera* (Pollen)	*Z. mays* (Pollen)
Egg	1.8 + 0.11	2.0 + 0.00	1.8 + 0.11	2.0 + 0.0
Larva	1.0 + 0.0	1.0 + 0.00	1.00 + 0.0	1.0 + 0.0
Protonymph	2.00 + 0.09 a	1.44 + 0.13 a	2.07 + 0.11 a	4.59 + 0.21 b
Deutonymph	2.47 + 0.16 a	1.81 + 0.10 a	2.13 + 0.13 a	6.0 + 0.42 b
Life cycle	7.27 + 0.15 a	6.25 + 0.11 a	7.00 + 0.13 a	13.25 + 0.59 b
% Survival	100	100	100	53
Preoviposition period	1.80 + 0.10	1.81 + 0.10	1.53 + 0.13	–
Oviposition period	37.67 + 1.09 a	20.68 + 1.25 b	38.27 + 1.16 a	–
Adult longevity	44.53 + 1.06 a	25.18 + 1.46 b	43.33 + 1.33 a	3.25 + 0.44 c
Life span	51.8 + 1.08 a	31.43 + 1.48 b	50.33 + 1.36 a	16.5 + 0.51 c
No. obs.	30	28	30	33

Different letters in a horizontal row denote significant difference (F test, $P< 0.05$)

Table 2

Effect of diet on the life table parameters of *E. yousefi* at 27 °C on test food substrates

Parameters	*E. dioscoridis*	*T. urticae* (Nymph)	*P. dactylifera* (Pollen)
Mean total fecundity (egg/♀)	63.8	50.5	57.60
Net reproductive rate (Ro)	47.4672	38.8290	37.0022
Generation time (days) (T)	16.6650	12.4089	16.8547
Intrinsic rate of natural increase (rm)	.2316	.2948	.2142
Finite rate of increase ($e^{r}m$), per day	1.2606	1.3429	1.2389
Sex ratio (females/total)	0.80	0.82	0.73

Discussion

The biology of *A. yousefi* has not been previously studied, but it may be important in regulating populations of the two spotted spider mite as well as eriophyid mite. Huffaker et al. (1969) discussed the divergence of the Phytoseiidae into specialized and generalized feeding strategies. Apparently, *A. yousefi* has evolved along the line of polyphagy since it can consume not only small animals, but also products of plant. Obligatory predation in larva of *E. yousefi* has been reported befor (Momen, 1999b) and other species, but it is not common among the species studied to date.

A number of authors have reported a superiority of the eriophyid mite *E. dioscoridis* over the tetranychid mite *T. urticae* as prey (Reda and El-Bagoury, 1986; Abou-Awad and El-Banhawy, 1986; Abou-Awad et al., 1989; Momen and El-Saway, 1993; Abou-El Ella, 1998; Abou-Awad et al., 1998; Momen, 1999a; Momen and Hussein, 1999). Eriophyids were easily consumed, probably favouring a high food conversion into egg biomass.

Pollen from different plant species do not have the same nutritional value and the intensity of pollen production may vary (Baker and Baker, 1979). It was evident from my investigations that date palm pollen promoted a higher reproductive rate of *A. yousefi* than did tetranychid prey. In contrast sweet corn pollen was unsuitable for development and reproduction in my study.

Pollen grains of sweet corn *Z. mays* was shown in laboratory studies to have high food value for *Euseius scutalis* A. H. (El-Badry and El-Banhawy, 1968), *Amblyseius hibisci* (Chant) McMurtry and Scriven, 1964), *Amblyseius largoensis* Muma (Kamburov, 1971). Surface structure of the pollen grain was thought to be one of the major factors affecting the feeding behavior of *Amblyseivs fallacis* (German) (Zhang and Li, 1989). The apple pollen grain, on a diet which *Euseius tularensis* (Congdon) showed very high rates of development and reproduction, has a thin exine (outer cell wall). The oat pollen grains, which were a poor diet for *E. tularensis,* have a thick wall (Kapp, 1969). According to the terminology of Overmeer (1985) the term "additional" or supplementary food was suggested to sweet corn pollen for this type of response. This supplemental foods may have value as a means of survival when suitable prey are not available (Caceres and Childers, 1991). It is worth to mention also that pollen grains is not a favorable food for egg production in *Amblyseius aleyredis* El-Badry (El-Badry, 1968), *Amblyseius lindquisti* Schuster and Pritchard (Momen, 1999a), *Typhlodromus transvaalensis* (Nesbitt) (Momen and Hussein, 1999).

The total number of eggs laid per female by *E. yousefi* seems to be in the higher range, respectively. Momen (1999b) reported that *E. yousefi* has a long adult period without reproductive activity on eggs of *T. urticae* and this implied that predation on eggs might not have sufficient prey for egg laying. Only a few species considered in the highest fecundity range, such as *Amblyseius bibens* Blommers or the genus *Phytoseius* Evans produce more than 50 eggs/female as well as *Neoseiulus californicus* Mcgregor (64 eggs/female) (McMurtry et al., 1970; Blommers, 1976; El-Laithy and El-Saway, 1998).

In the present study, the higher rm of *E. yousefi* reared on *T. urticae* could be attributed to differences in nutritive quality. Comparing the life table data of *E. yousefi* fed on *T. urticae* with those of other phytoseiids obtained at similar temperature and on the same prey species shows that *E. scutalis* had a lower (rm) and (Ro) (0.146–7.2) El-Laithy and Fouly, 1992). Tsolakis and Ragusa (1994) reported a lower value on *Amblyseius andersoni* (Chant) (rm = 0.178, Ro = 20.5) than in the present study. Excellent results were obtained by Takafuji and Chant (1976) with *Iphiseius degenerans* (Berlese) (rm = 0.248, T= 15.8, Ro = 50.9) and by Blommers (1976) with *A. bibens* (rm = 0.326, T= 12.0, Ro = 50.2).

The rm value of predacious mites are one of the criteria for evaluating the effectiveness of biological control agent of spider mites, but studies on the effects of environmental condition (Kashio and Tanaka, 1978) is thought to be necessary. Form this, it can be concluded *E. yousefi* that is promising biological control agent. Much more work has to be done on the responses of the predator to different insects and mite species, as well as plant products to clearify its role in biological control in greenhouses.

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Tables The title should be self-explanatory and include enough information so that each table is intelligible without reference to the text or other tables. The title should summarize the information presented in the table without repeating the subheadings. Subheadings should be brief (abbreviations are acceptable), nonstandard ones can be explained in footnotes. Cite tables in numerical order in the manuscript. Information presented in a table should agree with that in the text.

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Examples:

Érsek, T., Király, Z. and Dobrovolszky, A. (1977): Lack of correlation between tissue necrosis and phytoalexin accumulation in tubers of potato cultivars. J. Food Saf. 1, 77–85.

Deverral, B. J. (1992): Introduction. In: J. A. Bailey and J. W. Mansfield (eds): Phytoalexins. Blackie, Glasgow and London, pp. 1–20.

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