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Biochemical and Ecological Aspects of Plant-Parasite Relations

A Symposium Held on the Occasion of the 90th Anniversary of the Hungarian Research Institute for Plant Protection, Budapest, September 28—October 1, 1970

Edited by

Z. Király and L. Szalay-Marzsó

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The Relation of Polyphenoloxidase and Peroxidase to Symptom Expression in Tobacco var. "Samsun NN" after Infection with Tobacco Mosaic Virus

By

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Characteristic changes in the activities of polyphenoloxidase, peroxidase and catalase, and in the isoenzyme patterns of the former two enzymes on polyacrylamide gels were observed in Samsun NN tobacco plants inoculated with tobacco mosaic virus (TMV W U1). A new peroxidase isoenzyme with an optimum at low pH appeared by day 5. It reached maximal activity at day 6, and disappeared gradually thereafter. This isoenzyme was not present in the young leaves developing after infection, but total peroxidase activity was comparatively high in these tissues and correlated with systemic acquired resistance. In Samsun NN plants inoculated with TMV Holmes' ribgrass (TMV HR), no symptoms developed under the conditions used. Changes in polyphenoloxidase were similar to those found after inoculation with TMV W U1, but changes in peroxidase were markedly different. Peroxidase activity levelled off after day 3 and a new isoenzyme was not detected. Systemic acquired resistance was not achieved in this case. As injection with actinomycin D one day before inoculation resulted in both an increase in peroxidase activity and a decrease in lesion size, these experiments support the significance of peroxidase in the hypersensitive reaction of tobacco to TMV.

Increased activities of peroxidase and polyphenoloxidase have been recorded in TMV-infected tobacco plants, the increases paralleling symptom severity (FARKAS, KIRÁLY and SOLYMOSY, 1960; LOEBENSTEIN and LINSEY, 1966; SUSENO and HAMPTON, 1966). A role for polyphenoloxidase in the process of local lesion formation has been tentatively proposed (FARKAS, KIRÁLY and SOLYMOSY, 1960). However, increases in the activity of the enzymes did not appear to precede the appearance of visible symptoms. VAN KAMMEN and BROUWER (1964) reported, that polyphenoloxidase activity increased within a few hours after inoculation of "Samsun NN" tobacco plants with TMV, but this rise was not confined to the inoculated parts of the leaves. JOCKUSCH (1966) established that neither polyphenoloxidase activation nor the enzyme itself was sensitive to high temperatures *in vivo* as is the case with the hypersensitive reaction. CABANNE, SCALLA and MARTIN (1968, 1969) did not observe an increase in polyphenoloxidase activity before tissue necrosis developed, when systemically infected "Samsun NN" plants or "Xanthi nc." plants were transferred from 30 to 20°C. These findings make a cau-

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sal relationship between polyphenoloxidase activation and lesion formation seem unlikely. As soon as 1958, MARTIN had shown that in tobacco var. "Samsun", a rise similar to the one observed by VAN KAMMEN and BROUWER (1964) for TMV-inoculated "Samsun NN" plants, occurred after infection. In this variety, TMV induces systemic mosaic symptoms. It should also be noted that such initial rises are surpassed later during pathogenesis when symptoms are fully expressed. Under such conditions, polyphenoloxidase activity in TMV-infected "Samsun" plants showing no necrosis may significantly exceed the level of activity in "Samsun NN" plants at the appearance of necrotic lesions.

Furthermore, it has become clear that the virus is not localized by the polyphenol barrier, but is present in an active state in a ring around the lesion (HAYASHI and MATSUI, 1965). This is also evidenced by the fact that transfer of infected plants to 30°C brings about mosaic symptoms and virus multiplication in the uninoculated upper leaves. Hence, it seems unlikely that toxic quinones produced by polyphenoloxidase action, can be responsible simultaneously for collapse of cells at the lesion edge and localization of the virus several layers of cells beyond. A reinvestigation of the role of polyphenoloxidase seemed worthwhile. On the other hand, peroxidase has been implicated as an important factor in resistance of tobacco to *Pseudomonas tabaci* (LOVREKOVICH, LOVREKOVICH and STAHMANN 1968a) and in aspecific resistance to TMV-induced local lesion formation in "Samsun NN" plants previously infected with this bacterium (LOVREKOVICH, LOVREKOVICH and STAHMANN 1968b). Therefore, the role of peroxidase was given special attention.

Materials and Methods

Experiments were performed using 10–12 week old tobacco plants (*Nicotiana tabacum* L.) var. "Samsun NN" grown as described previously (VAN LOON and VAN KAMMEN, 1968). All green leaves of the plants were inoculated either with a U1 strain of TMV, designated W U1 (VAN LOON and VAN KAMMEN, 1970) or the Holmes' ribgrass (HR) strain (HOLMES, 1941). The W U1 strain induced local lesions on the inoculated leaves. With the HR strain, small necrotic lesions on the inoculated leaves were obtained on young plants under greenhouse conditions, but no symptoms developed when plants were kept in a growth chamber under the conditions used in the present experiments: light intensity 25,000 erg sec.$^{-1}$ cm^{-2} at plant height; temperature 18–20°C; relative humidity not less than 70 per cent. No virus could be recovered from plants kept under these conditions.

For comparison, tobacco plants var. "Samsun" were also used in some cases. Systemic mosaic symptoms appeared on the young, developing leaves in this combination 10–14 days after inoculation with TMV W U1. Water-inoculated plants served as controls in all experiments.

Up to seven days after inoculation, samples were taken daily from identical leaf positions with regard to the tips of the plants. Centrifuged extracts were pre-

pared according to VAN LOON and VAN KAMMEN (1968). Samples of the extracts were subjected to polyacrylamide gel electrophoresis in the gel system of DAVIS (1964) at pH 9.5 with minor modifications (VAN LOON and VAN KAMMEN, 1968). Development of the isoenzyme patterns of polyphenoloxidase and peroxidase with caffeic acid and guaiacol as the substrate and hydrogen donor, respectively, were performed as described earlier (VAN LOON, 1971). The remainder of the extracts was dialysed overnight and subsequently used for determination of polyphenoloxidase, peroxidase, and catalase activity. Activities were measured spectrophotometrically; polyphenoloxidase with chlorogenic acid as the substrate (VAN KAMMEN and BROUWER, 1964), and peroxidase with guaiacol as the hydrogen donor (TOMIYAMA and STAHMANN, 1964). Catalase activity was determined by following the decrease in absorption at 240 nm after addition of hydrogen peroxide to the enzymic solution (BEERS and SIZER, 1952).

Results and Discussion

Figure 1 shows the relative activity of polyphenoloxidase at successive days after inoculation as compared to the water-inoculated control. A rise in polyphenoloxidase activity appeared in TMV W U1-inoculated "Samsun NN" plants during the first two days. A nearly identical rise was observed in "Samsun NN" plants inoculated with TMV HR, although no symptoms would develop in the latter combination. A similar rise in polyphenoloxidase activity was also observed in "Samsun" plants during the first days after inoculation with TMV W U1. This confirmed the results obtained by MARTIN (1958). It seems, therefore, that the first peak in polyphenoloxidase activity does not bear a causal relationship to lesion formation.

After inoculation of "Samsun NN" plants with TMV W U1, local lesions appeared within 50–60 hours. The appearance of symptoms was followed by a sharp decrease in polyphenoloxidase activity. Subsequent enlargement of lesions was observed up to day 6, coinciding with a progressive increase in polyphenoloxidase activity. After lesion enlargement had stopped, activity rapidly decreased to normal levels. TMV HR-infected plants showed a similar reaction up to day 4, after which levels became normal. The second peak in polyphenoloxidase activity was thus clearly influenced by the presence or absence of visible local lesions. This may reflect that once lesions had developed, a decrease of polyphenoloxidase activity was prevented through progressive necrosis of cells at the lesion edges. Combined with the fact, that viral multiplication and migration precedes lesion development, this would indicate that the increase in polyphenoloxidase activity is a secondary phenomenon, comparable to the generally observed rise in polyphenoloxidase activity after wounding.

During seven days after inoculation, neither qualitative differences, nor changes in the relative proportions of the polyphenoloxidase isoenzyme bands

were detected by polyacrylamide gel electrophoresis, as shown in Fig. 2 for 5 day TMV W U1-infected plants. This observation confirms the conclusion of VAN KAMMEN and BROUWER (1964), reached by comparing K_m values of the enzyme in

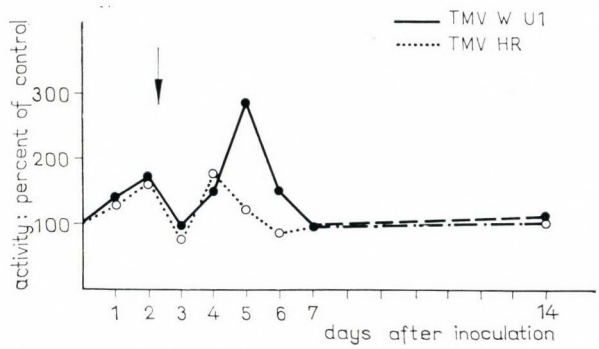

Fig. 1. Relative polyphenoloxidase activities in "Samsun NN" tobacco plants after inoculation with TMV W U1 or TMV HR at a concentration of 1 μg per ml. Polyphenoloxidase activity was measured according to VAN KAMMEN and BROUWER (1964) with chlorogenic acid as the substrate at pH 5.5. The activity measured in water-inoculated plants was taken as 100 for every day. The values at day 14 refer to activities in young leaves developed after inoculation. The arrow marks the appearance of visible symptoms in TMV W U1-inoculated plants

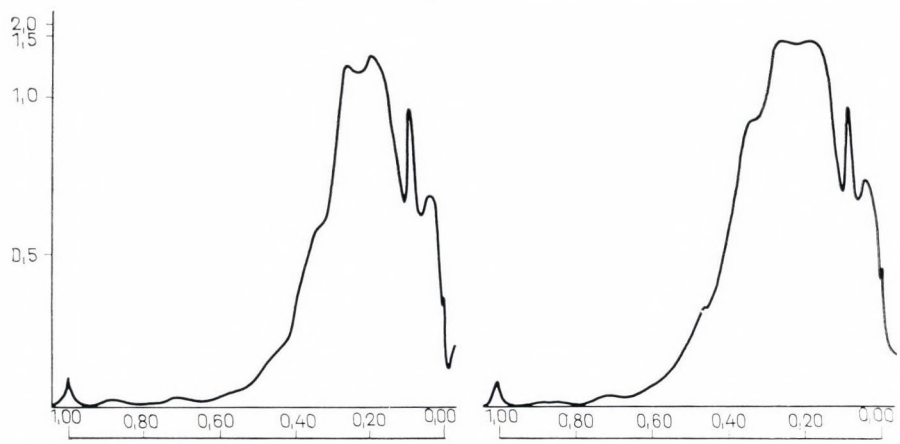

Fig. 2. Densitometer tracings of polyphenoloxidase isoenzyme patterns of (left) water-inoculated and (right) 5 day TMV W U1-infected "Samsun NN" tobacco plants on 7.5 per cent polyacrylamide gels. Patterns were developed with caffeic acid and *m*-phenylenediamine at pH 5.5 (VAN LOON, 1971)

non-infected and infected plants, that no new polyphenoloxidase isoenzyme would be induced upon infection.

Newly formed leaves from plants inoculated with TMV W U1 14 days before, exhibited strong systemic acquired resistance (ROSS, 1961b), as manifested

by the small size of the lesions which appeared after challenge inoculation with the same virus strain. However, comparable leaves from TMV HR-inoculated plants did not show systemic acquired resistance. In both instances, these leaves showed normal polyphenoloxidase activity (Fig. 1). When such leaves from plants previously inoculated with TMV W U1 were challenge inoculated, and polyphenoloxidase activity was subsequently measured during four days, a pattern similar

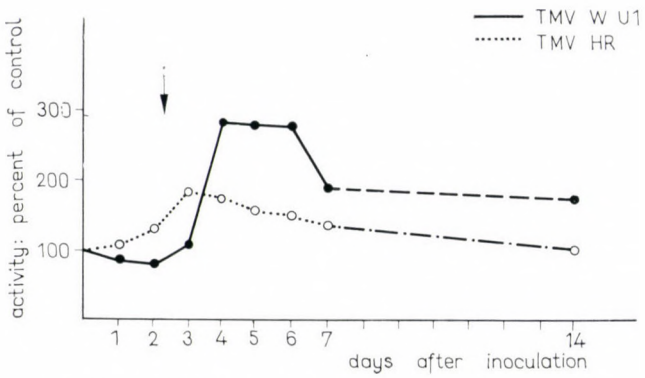

Fig. 3. Relative peroxidase activities in "Samsun NN" tobacco plants after inoculation with TMV W U1 or TMV HR at a concentration of 1 μg per ml. Peroxidase activity was measured according to TOMIYAMA and STAHMANN (1964) with guaiacol as the hydrogen donor at pH 5.5

to that of Fig. 1 emerged. Polyphenoloxidase levels did not exceed those attained after the first inoculation and showed only a 25 per cent increase in activity over the control by day 4. This indicates that polyphenoloxidase cannot be responsible for the expression of systemic acquired resistance. The rise in activity commencing after day 3 seems related to the amount of tissue necrotizing at that stage. These observations therefore support the conclusion that stimulation of polyphenoloxidase activity is a secondary phenomenon.

Changes in peroxidase activity at successive days after inoculation are shown in Fig. 3. In TMV W U1-infected plants an initial decrease lasting for two days was followed by a sharp rise at symptom appearance. The activity remained high after day 6 when lesions did not enlarge any more. Plants inoculated with TMV HR showed a moderate but progressive increase until the third day after inoculation. After this the activity gradually levelled off. Newly developed leaves from plants inoculated with TMV W U1 14 days before still showed increased peroxidase activity, whereas such leaves from plants previously inoculated with TMV HR had normal peroxidase levels. These leaves from TMV W U1-inoculated plants showed systemic acquired resistance, whereas comparable leaves from TMV HR-inoculated plants did not. Leaves from 7 day TMV HR-inoculated plants showed some local acquired resistance (ROSS, 1961a). After challenge inoculation with TMV W U1, lesion diameters were reduced not more than ten per cent as compared to those on plants which had been previously inoculated with water.

Polyacrylamide gel electrophoresis patterns of peroxidases from water-inoculated and 6 day TMV W U1-infected plants are shown in Fig. 4. At this stage all bands which are present in non-infected leaves and noticeably those with R_f values 0.32 and 0.56 were more intense in infected plants. In addition, a new band with R_f 0.12 was detected in infected leaves. This isoenzyme was clearly discovered only when guaiacol was used as the hydrogen donor and gels were examined for

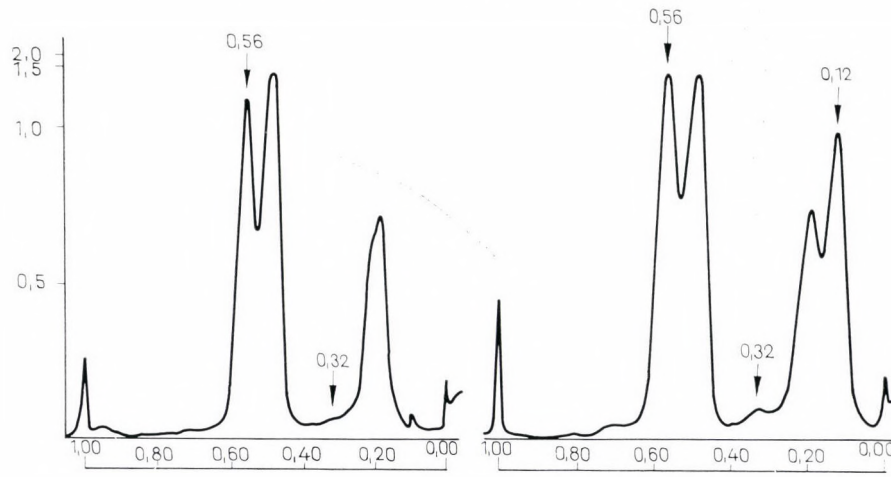

Fig. 4. Densitometer tracings of peroxidase isoenzyme patterns of (left) water-inoculated and (right) 6 day TMV W U1 infected "Samsun NN" tobacco plants on 7.5 per cent polyacrylamide gels. Patterns were developed with hydrogen peroxide after preincubation with guaiacol at pH 5.5 (VAN LOON, 1971)

peroxidases at acidic pH values. It readily developed at pH 2.5 or after stopping the reaction with 7 per cent acetic acid. A rise in all bands normally present coincided with the rise in peroxidase activity three days after inoculation. Band 0.12 appeared only at day 5, marking the moment when lesion enlargement was almost terminated. As judged from the patterns, the increases in all other bands became smaller after day 4. On the other hand, the new isoenzyme reached maximal activity at day 6, and disappeared gradually thereafter. Its concentration seemed dependent on lesion density as in extracts from leaves inoculated with 100 μg TMV per ml colour intensity in this band greatly surpassed that of all others. The new isoenzyme was not present in young leaves developing after inoculation, but was induced after challenge inoculation with TMV W U1, appearing again about five days after the second inoculation.

The early rise in peroxidase activity in TMV HR-inoculated plants was connected with an increase in the activity of the peroxidase bands normally present. The new peroxidase isoenzyme was not induced at any time in this combination.

TMV W U1 challenge inoculated young leaves from plants previously infected with the same strain were assayed for peroxidase activity during four days. Activity followed a pattern different from that of Fig. 3, in that peroxidase was activated about 50 per cent during the first three days. As activity was already nearly 100 per cent higher at the moment of challenge inoculation, this means that much higher peroxidase levels were attained earlier after the second inoculation than after the first one.

These observations indicate that a new peroxidase isoenzyme is induced after appearance of local lesions. Once lesions have developed, this isoenzyme might play a role in limiting further spread of the virus. However, systemic acquired resistance is expressed in its absence. Peroxidase activity is comparatively high in the young leaves, which develop after infection. Therefore, *high peroxidase activities might reduce the rate of lesion enlargement and thus be responsible for the expression of systemic acquired resistance.*

The mechanism of local lesion formation in leaves exhibiting systemic acquired resistance is considered not to be different from that in leaves inoculated for the first time (Ross, 1966; Davis and Ross, 1968; Ross and Israel, 1970). This hypothesis finds support in observations that activities and isoenzyme constitution of both polyphenoloxidase and peroxidase followed similar tendencies in both cases. However, *peroxidase levels were much higher and activation of this enzyme occurred earlier in leaves showing acquired resistance.* Thus, the state of acquired resistance seemed correlated with high peroxidase levels. A similar conclusion was recently reached by Simons and Ross (1970) when these authors followed peroxidase activity and development of acquired resistance in TMV-infected "Samsun NN" plants.

It became of interest to study also changes in catalase activity during pathogenesis. On the one hand, catalase will compete with peroxidase for the substrate, hydrogen peroxide, while on the other hand it may also act in a peroxidatic way itself (Mahler and Cordes, 1967).

Figure 5 shows that both after inoculation with TMV W U1 or TMV HR a high rise in catalase activity was observed on day 1. In TMV W U1-infected "Samsun NN" plants catalase levels fell off rapidly after the initial rise. Low levels were present from the third day on. A similar, but less pronounced pattern was also observed in "Samsun" plants after inoculation with TMV W U1. However, "Samsun NN" plants inoculated with TMV HR showed a second, extremely high peak at day 3, after which levels gradually returned to normal. Whereas in TMV U1-infected plants relatively low peroxidase activities were accompanied by high catalase levels and high peroxidase levels were associated with low catalase activities, plants inoculated with TMV HR were characterized by both peroxidase and catalase levels above normal during the initial stage. The second catalase peak coincided with the end of the increase in peroxidase activity, possibly marking the moment plants had recovered from their interaction with the inoculated virus.

Newly formed leaves from plants inoculated with TMV W U1 14 days before,

also showed both high catalase and high peroxidase levels (Fig. 5). When such leaves were assayed for catalase activity after challenge inoculation with the same virus strain during four days, catalase activity was slightly decreased by day 1, but showed levels exceeding those of control plants during the following, days. Hence, resistance may also involve catalase. However, in view of its different possible modes of action, a coherent picture can not be given at present.

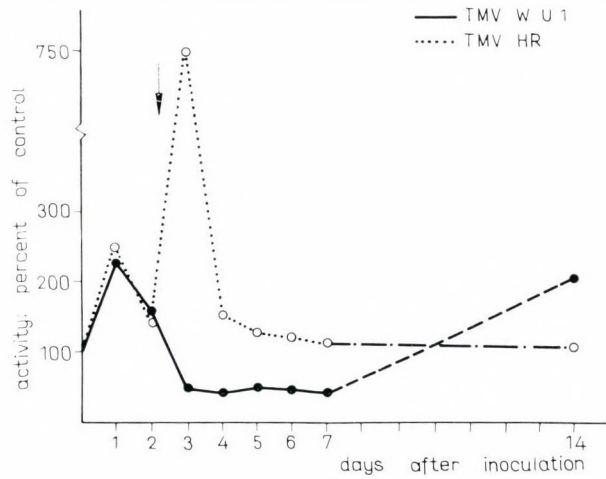

Fig. 5. Relative catalase activities in "Samsun NN" tobacco plants after inoculation with TMV W U1 or TMV HR at a concentration of 1 μg per ml. Catalase activity was measured according to BEERS and SIZER (1952) at pH 5.5

The possible significance of peroxidase in the hypersensitive reaction of tobacco to TMV was further investigated. As actinomycin D (AMD) inhibits DNA-dependent RNA-synthesis without inhibiting the multiplication of TMV-RNA (SÄNGER and KNIGHT, 1963), this antibiotic seemed suitable for studying the nature of both peroxidase activation and symptom expression without interfering with virus synthesis. From "Samsun NN" plants all leaves below the last three fully expanded leaves were removed. Into the remaining leaves AMD at a concentration of 20 μg per ml was injected with a hypodermic needle (KLEMENT, 1963; LOEBENSTEIN, RABINA and VAN PRAAGH 1968; VAN LOON and VAN KAMMEN, 1970). Leaves were injected with AMD either one day before or two or four days after inoculation with water or TMV W U1. As AMD seems to be active in tobacco plants for three days (LOEBENSTEIN, RABINA and VAN PRAAGH, 1968), leaves were extracted and assayed for enzyme activities and isoenzyme patterns at this time. Leaves injected with water served as a control in all experiments.

Results are presented in Table 1. In every case peroxidase activity appeared to be increased about 50 per cent by treatment with AMD. Activity in all bands

present had increased roughly to the same extent as shown by polyacrylamide gel electrophoresis. This indicated that no activation of specific isoenzymes had occurred. On the other hand, no effect of AMD on polyphenoloxidase activity was found. Catalase activity was inhibited about 30 per cent in every combination.

When AMD was injected one day before inoculation, lesion diameters were slightly reduced. No significant differences in lesion size were detected when the treatment with AMD was made two or four days after inoculation. This can be

Table 1

Effect of Actinomycin D on peroxidase activity*

Time of injection days	Time of inoculation days	Time of extraction days	Relative peroxidase activity** in leaves			
			water-injected water-inoculated	AMD-injected water-inoculated	water-injected TMV-inoculated	AMD-injected TMV-inoculated
−1	0	2	100	149	102	157
2	0	5	100	155	232	254
4	0	7	100	150	202	292
non-inoculated developing leaves of plants inoculated at day 0						
13	14	16	100	138	75	124

* Leaves were injected with water or AMD at a concentration of 20 μg per ml, and inoculated with water or TMV W U1 at a concentration of 100 μg per ml.

** Activities were measured as described in the legend of Fig. 3. The activity measured in water-injected, water-inoculated leaves was taken as 100 for every series.

understood if factors other than peroxidase are also responsible for inhibition of lesion enlargement. This pertains especially to the later stages of lesion development. The limiting action of peroxidase on lesion enlargement might be counteracted by a reduction in the amount of other inhibiting factors, which depend on DNA-dependent RNA-synthesis. Clearly, the four new protein components detected after lesion development in TMV W U1 infected "Samsun NN" plants (VAN LOON and VAN KAMMEN, 1970) might fulfill such requirements.

In leaves showing systemic acquired resistance, AMD significantly reduced lesion diameters when injected one day before challenge inoculation (Table 2). Thus, it is clear that *treatment with AMD did not increase lesion diameter as compared to the water-injected controls.* These results are in contrast to the conclusion drawn by LOEBENSTEIN and co-workers (1968; 1969) that "AMD applied close in time to the inoculation partially inhibits the formation of the mechanism responsible for the localization of the infection in hypersensitive hosts".

The effect of AMD on lesion diameter is also expressed when detached leaves are inoculated with TMV after taking up AMD through their petioles, using the technique of SÄNGER and KNIGHT (1963). In detached leaves, local lesions

Table 2
Lesion diameters in Samsun NN tobacco leaves exhibiting systemic acquired resistance*

Treatment	Diameter (mm)
Water-injected, TMV-inoculated	0.45±0.17
AMD-injected, TMV-inoculated	0.40±0.13**

* Leaves were injected with water or AMD at a concentration of 20 μg per ml and inoculated with TMV W U1 at a concentration of 10 μg per ml. 79 and 55 lesions, respectively, were measured at two axes perpendicular to each other, five days after inoculation.

** Significant at the 2.5 per cent level ($p<0.025$).

continue to increase resulting in greater differences in size between lesions on water-treated or AMD-treated leaves (VAN LOON, unpublished results). Figure 6 shows that lesion diameters were decreased by treatment with increasing concentrations of AMD. This effect was even more pronounced when leaves were kept at 30°C during AMD uptake and inoculation with TMV, and transferred to 20°C one day later. The mechanism of local lesion formation is not operative at 30°C. Therefore, these results provide proof that the hypersensitive reaction can not be inhibited by AMD and thus can not be directly dependent on the DNA-directed synthesis of proteins that inhibit lesion enlargement, as suggested by LOEBENSTEIN et at. (1968, 1969). Instead *the hypersensitive response was found to be stimulated by AMD.* This might be explained by assuming that genes which are re-

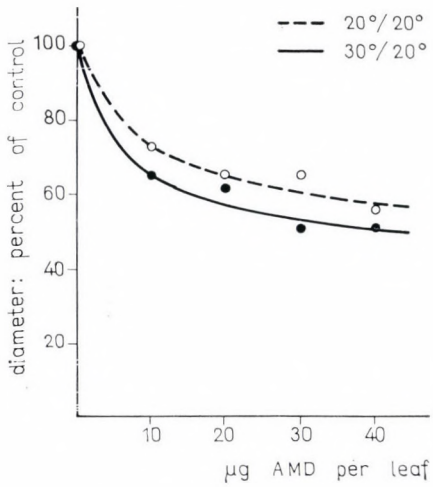

Fig. 6. Relative lesion diameters, measured 4 days after inoculation of detached leaves of "Samsun NN" tobacco plants with TMV W U1 in dependence of the amount of actinomycin D taken up by individual leaves before inoculation

sponsible for synthesis of macromolecules involved in the hypersensitive reaction are repressed under normal conditions. AMD then might interfere with the repressing mechanism.

Such a mechanism would not be unique, as it has been shown that the synthesis of pisatin, a phytoalexin produced by incubation of pea pod tissue with a spore suspension of the fungus *Monilia fructicola*, is induced by AMD (SCHWOCHAU and HADWIGER, 1968). To account for this observation, a similar hypothesis has been advanced for this system (SCHWOCHAU and HADWIGER, 1969).

In view of the fact that *AMD stimulates both the hypersensitive reaction and peroxidase activity*, but not polyphenoloxidase and catalase activity in TMV W U1 infected "Samsun NN" tobacco plants, the significance of peroxidase in limiting lesion enlargement in hypersensitive hosts seems clearly established.

*

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Biosynthesis of Phenylpropanoids and Coumarins in TMV-infected Tobacco Leaves and Tobacco Tissue Cultures

By

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Habituated tobacco tissue cultures derived from stems contain high amounts of chlorogenic acid and coumarins. However, infection of the cultures with TMV does not change the level of these compounds significantly. On the contrary, TMV-infection of tobacco leaves induces important changes in the production of phenolics especially in the case of the necrotic hosts. Using *Nicotiana tabacum* var. Samsun NN, a necrotic host, studies have been conducted on the effect of infection by TMV on the labelling of the phenylpropanoids from (U)-^{14}C-phenylalanine.

It has been known for some time that appearance of unusual phenolic compounds and increased accumulation of others normally found in low concentration occur in many plants infected by various phytopathogens [1, 2]. In that situation, the more frequently encountered compounds are chlorogenic acid (I) and various coumarin derivatives such as umbelliferone (II) and scopoletin (III) (Fig. 1). For instance the latter compound, scopoletin. has been found to accumulate in potato infected with *Ceratocystic fimbriata* [3] and with *Phytophtora infestans* [4] in tobacco infected with *Pseudomonas solanacearum* 5 and with various viruses [6–8]: thus, it is clear that increase in the levels of phenolic compounds is not a specific response of a given host towards a given parasite.

The present work deals with tobacco mosaic virus (TMV) infections of tobacco tissues. Most strains of TMV produce a systemic mosaic disease in the majority of tobacco varieties. However, some varieties react to certain virus strains in a different way: instead of systemic spread, virus multiplication leads to the formation of necrotic lesions at the points of infection and a limitation of virus spread occurs in the vicinity of these lesions. Moreover the accumulation of phenolic compounds is higher in the case of the latter type of viral infection than during systemic spread of the virus. It has been believed during some time that the simultaneous increases in the levels of polyphenolic compounds and also in polyphenoloxidase [9] and peroxidase activities are related to the inhibition of virus spread: increased production of oxidation compounds toxic to the cells would lead to the formation of a barrier of dead cells, and this might explain why virus cannot spread any more. But it is well established now that the necrotic lesions are surrounded by living cells containing infectious virus particles. Thus, it is still

not clear how phenolics are involved in the reaction of hosts towards parasites. Moreover, very little is known about their biosynthesis in diseased tissue.

The present paper reports the results of studying the biosynthesis of scopoletin (III), scopolin (IV) and chlorogenic acids (I) in TMV infected tobacco tissues. The following objectives were considered:

1) Relationship between the metabolism of phenolic compounds and the formation of necrotic lesions. Results of experiments are described, in which healthy and TMV infected leaves of a hypersensitive tobacco variety were fed with ^{14}C-labelled phenylalanine.

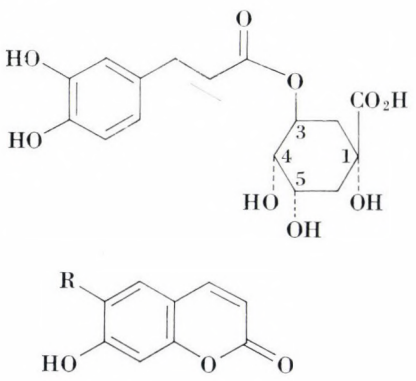

Fig. 1

(I) Chlorogenic acid
Coumarin derivatives:
(II) umbelliferone (II) R = H
(III) scopoletin (III) R = OCH_3
(IV) scopolin, monoglucoside of scopoletin

2) Is the rise in the levels of phenolic compounds a general response to TMV infection of tobacco tissues? Results obtained with TMV infected tobacco leaves and habituated tissue cultures are compared.

Materials and Methods

Plant material. The tobacco tissue cultures used in these experiments have already been described [19, 20], as well as their use in biosynthetic investigations [13]. Tobacco plants, variety "Samsun NN", were grown in a greenhouse at 22 ± 1°C. Four weeks old plants with 6 leaves were used in the virus inoculation and feeding experiments.

Virus inoculation. The upper side of the leaves was rubbed in the presence of Celite with a 20 μg/ml solution of TMV.

Feeding experiments. A solution containing mineral salts (the solution of Knop[19] with a three fold dilution) and (U)- ^{14}C-phenylalanine (specific activity: 130 mC/mM) was fed to the leaves by uptake through the cut petiole. About 20 ml of solution containing 20 μC of phenylalanine were usually taken up by 30 to 40 g fresh weight of leaves within 2 1/2 hours.

Isolation of compounds. Leaves were ground in a Waring blendor and extracted with several portions of hot methanol. After filtration, water was added and the solution allowed to stand one night in order to precipitate most of the chlorophyll.

For isolation of both free and bound phenolic acids classical paper chromatography was performed. [10, 11, 12, 15, 21–24] Scopoletin and scopolin were isolated by successive paper chromatographies (with *n*-butanol–ethanol–water, 4/1/2, and then benzene–acetic acid–water, 6/7/3 for the former and *n*-butanol–acetic acid–water, 4/5/1 for the second). Scopolin was then hydrolysed to scopoletin with 4 *N* HCl. Final purification was achieved by paper chromatography with a 2% solution of acetic acid in water.

Quantitative determinations. Phenylpropanoids were determined by u.v.: spectrophotometry. Scopoletin and scopolin were determined by u.v. spectrophotometry or, when present in very low amounts, with an Aminco-Bowman spectrophotofluorimeter.

Measurements of radioactivity. Countings were performed with a scintillation spectrometer. Bray's solution (with dioxan as the basic solvent) was used as scintillation liquid [25].

Results

A. *Biosynthesis of phenylpropanoids and coumarins in TMV infected leaves of the hypersensitive tobacco variety "Samsun NN"*

TMV infected leaves of a hypersensitive tobacco variety accumulate more phenolic compounds than leaves giving a systemic virus spread. This has been confirmed very recently by the work of TANGUY and GALLET [10–12] who investigated the phenolic content of healthy and infected leaves of *Nicotiana tabacum* variety "Xanthi" and of *Nicotiana tabacum* variety "Samsun". We summarize their results : infection at 20°C of "Xanthi leaves" with the wild strain of TMV leads to the formation of necrotic lesions whereas infection of "Samsun" leaves with the same virus strain results in a systemic spread of the parasite. However, "Xanthi" leaves infected at 30°C show also a systemic infection by the TMV wild strain. Comparison of the phenolic levels measured in the two tobacco varieties after various infection periods and under various temperature conditions enabled these authors to draw the following main conclusions:

1) The increased accumulation of phenolics occurs when the experimental conditions allow the formation of local necrotic lesions.

2) Significant accumulation appears only when once the necrotic lesions are visible.

In fact, concentration determinations without the isotope dilution method are not very accurate because of the many steps necessary in the purification of the compounds. Moreover, such measurements are not a sensitive mean to estimate low quantitative changes, especially at the early stages of infection, when only a few cells are invaded by the parasite.

These difficulties may be overcome by the use of radiochemical methods. The label measured for a compound corresponds to its synthesis during the feeding period, and thus, the sensitivity of this type of determination is not affected by the endogenous pool of substance already present before the feeding. Moreover, the knowledge of the specific radioactivity of a compound and of the rate of incorporation of the precursor into it gives good information about the dynamical aspect of its biosynthesis, i.e. its turnover rate [13]. Our purpose was to detect changes in the biosynthesis at the early stages of infection and just before as well as after the formation of necrotic lesions.

Healthy "Xanthi" leaves do not contain detectable amounts of scopoletin and scopolin. Since we wished to follow with time the specific radioactivity of these compounds from the beginning of the infection, we used *Nicotiana tabacum* plants, variety "Samsun NN".

This hypersensitive variety is similar to the "Xanthi" variety, however it contains measurable amounts of the two coumarins. Only one phenylpropanoid is easily detectable in the healthy leaves of both varieties: caffeic acid. This hydroxycinnamic acid is present mainly as 3−0-caffeoylquinic acid (chlorogenic acid) and to a lower extend as 4−0-caffeoyl-quinic and 5−0-caffeoylquinic acids.

Results of feeding the plants after the appearance of local lesions. "Samsun NN" leaves were rubbed with a solution containing Celite and TMV wild strain at a concentration of 20 μg/ml. Experiments carried out between January and June 1970 with plants of the same age showed that the first visible lesions on the mid-leaves always appeared after a period of 40 $\pm$ 2 hours following inoculation.

Table 1 summarizes the results obtained in a typical experiment involving both virus inoculation and ^{14}C-phenylalanine feeding. 46 hours after inoculation the mid-leaves showed visible lesions and were cut from the stems. A solution containing mineral salts and the radioactive precursor was fed to the leaves by uptake through the cut petiole. Controls consisted of leaves rubbed with Celite only, cut after 46 hours and fed in the same way as the infected leaves. As can be seen from Table 1, after a 48.5 hours (46 + 2.5) infection, there is a sharp increase in the concentrations of the 3 compounds tested. It must be pointed out that in this particular experiment healthy leaves contained unusually low amounts of chlorogenic acid. Infection leads usually to a 3−5 fold increase in chlorogenic acid and a 10−20 fold increase in scopoletin and scopolin. But this extreme case shows even better that the difference in the concentration of the three compounds lies in a tremendous stimulation of their biosynthesis. Since it has been shown that

scopoletin and chlorogenic acid have rather high turnover rates [13] and since very little is known about their routes of catabolism, accumulation could equally well arise through a strong decrease of their rate of catabolism and through similar rates of synthesis. This is not the case, as shown by the strong increase in the incorporation rates, especially into caffeic acid. Since phenylalanine is not a specific precursor of the phenylpropanoids, the 17% incorporation into caffeic acid is surprisingly high and indicates important modifications in the use of phenylalanine by infected leaves, when compared to its use by healthy leaves.

Table 1

^{14}C-phenylalanine feeding experiment following a 46-hour-infection period

Compounds	Healthy leaves (38 g fresh weight)			TMV-infected leaves (36 g fresh weight)		
	Concentration (μg/g fresh weight)	Dilution value	Incorporation (rate) %	Concentration (μg/g fresh weight)	Dilution value	Incorporation (rate) %
Scopoletin	0.08	250	0.03	1.0	250	0.4
Scopolin	1.5	300	0.25	39	800	2.5
Chlorogenic acid	15	4500	0.18	650	2100	17

Both healthy and infected leaves were fed with 25 μC of (U)-^{14}C-phenylalanine(130 mC/mM) over a period of *150 minutes*.

Concerning the other phenylpropanoids, we experienced a low increase in the label of *p*-coumaric and ferulic acids. Since ferulic acid is a precursor of scopoletin and scopolin [13–15] this result shows that infection leads to a simultaneous stimulation of O-methylation and cyclisation activities.

Biosynthesis of chlorogenic acid and coumarins at the early stages of infection. We carried out 5 parallel experiments with plants of the same age and from the same batch. Our purpose was to answer the following questions: is the stimulation of the biosynthesis detectable just after inoculation. Is it progressive? Does it start already before the local necrotic lesions are visible?

In 4 experiments leaves were rubbed with Cellite and TMV 6 hours, 12 hours, 24 hours and 36 hours respectively before a 150 minutes feeding with ^{14}C-phenylalanine. In the control experiment leaves were rubbed with Celite 24 hours before the feeding. The amounts of scopoletin, scopolin and chlorogenic acid were approximatively the same in the 5 methanolic extracts. The measured specific radioactivities are given in Table 2. *Surprisingly there is no significant change in the biosynthesis of the three compounds with time of infection.* The 2.5 fold higher values obtained in the first experiment (6 hours of infection) probably correspond to a response to the recent wounding of the leaves. In the case of all parallel experiments, infected leaves showed visible lesions approximatively 40 hours following

inoculation. Table 2 shows that after 36 hours there is still no significant increase in the biosynthesis of the tested compounds. Of course, more work is necessary to determine the exact time of stimulation and the sequence in the increase of the different involved enzymatic activities: phenylalanine deaminase, cinnamic acid hydroxylase, phenolase, O-methyl transferase, cyclase. However, it is already possible to conclude that the increase in the rate of synthesis of these compounds is not progressive; on the contrary, it starts intensively at a time very close to that of lesions appearance.

Table 2

Biosynthesis of chlorogenic acid and coumarins at the early stages of infection

Infection period	Fresh weight (g)	Absorbed radioactivity (μC)	Specific radioactivities (10^5 dpm/μM)		
			Scopoletin	Scopolin	Chlorogenic acid
6 hours	43.1	19.9	17.8	13.0	1.1
12 hours	44.5	19.9	8.3	5.8	0.40
24 hours	42.8	19.9	9.0	6.4	0.38
36 hours	41.0	18.3	7.9	4.5	0.40
Controls (24 hours)	42.0	17.9	6.8	4.6	0.35

Infection periods precede the feeding experiments. Control leaves were rubbed with Celite 24 h before feeding. The cut leaves were fed with 20 μC of (U)-^{14}C-phenylalanine (130 mC/mM) for *150 minutes*.

Furthermore, since it is now well established that uncoating of the TMV proceeds within a few hours, it appears that there is no direct relationship between virus multiplication which starts immediately after uncoating and accumulation of phenolic compounds which occurs almost two days later. The increase in the biosynthesis of these compounds is probably to be related to a genetic derepression of their metabolism as a result of infection.

B. *Phenylpropanoids and scopoletin levels in healthy and TMV infected tobacco tissue cultures*

Tissue cultures have been used very little for the study of both virus multiplication and the biosynthesis of phenolic compounds. Since this material consists of undifferentiated cells and has no organs, biosynthetic investigations are easier to carry out: there is no problem of mechanical transport of precursors, as in whole plants; it remains only the problem of transport across a cell wall. Consequently, incorporation times can be kept very short and thus the main pathway is easier to find: the label appears first in compounds of high turnover rate [13].

We studied the biosynthesis of scopoletin in tobacco tissue cultures [13] which contain large amounts of this coumarin, mainly as its monoglucoside. Since this

phenolic compound is one of the most frequently encountered in the host reaction to parasitic disease, we measured its levels in healthy and TMV-infected tobacco cultures. Table 3 shows that infection does not lead to an increased amount of scopoletin in infected material: sometimes levels are a little bit higher in healthy

Table 3

Total scopoletin amounts (μg/g fresh weight) in 40 days old healthy and TMV infected tobacco tissue cultures

Experiments	Not infected cultures	TMV infected cultures
I	88	57
II	55	45
III	57	62

These tissue cultures were grown on a basic mineral medium whithout auxin and kinetin.

Table 4

Evolution of scopoletin and scopolin amounts with time of growth in healthy and TMV infected tobacco tissue cultures*

	Not infected cultures				TMV infected cultures			
Age of cultures (days)	10	20	30	60	10	20	30	60
Scopoletin (μg/g fresh weight)	1.6	1.1	2.5	6.4	0.58	0.46	0.56	1.5
Scopolin (μg/g fresh weight)	83	87	106	168	67	58	80	104

* Tobacco tissue cultures were grown on the medium of Murashige and Skoog.

tissue, sometimes the reverse situation occurs. Table 4 indicates results obtained with tobacco tissue cultures growing on the medium of SKOOG and MURASHIGE [17]. These cultures show some resistance to the parasite: after many transfers (about 15 to 20) the concentration of virus decreases and it is sometimes possible to obtain virus-free cultures [18]. It is seen in Table 4 that infected cultures contain even lower amounts of scopoletin and scopolin than healthy cultures. But, as shown by other measurements at various ages of the cultures, the reverse situation may occur as well: finally no reproducible difference is detectable. Certainly, one can argue that tissue cultures contain much less virus than the infected leaves, and thus it is not reasonable to compare their reaction as hosts. In fact, cells of tissue cultures contain very much inert material such as cellulose and lignins. When the virus concentration is related to the protein content, it appears that tissue cultures contain also much virus.

Conclusions

These results obtained with tissue cultures confirm those found with infected leaves: there is no direct relationship between virus multiplication and levels of phenolic compounds. In tobacco leaves virus multiplication leads to a strong stimulation of the biosynthesis of phenolic compounds whereas in tobacco tissue

Table 5

Comparison of the hydroxycinnamic acids and scopoletin content of healthy and TMV infected tobacco leaves (variety "Samsun NN") and tobacco tissue cultures

Compounds	Healthy leaves	TMV infected leaves	Healthy and TMV infected tissue cultures
p-Coumaric acid	+	+	+
Caffeic acid	+++	+++	+++
Ferulic acid	+	+	++
Scopoletin	+	+++	+++

Key: +, in small amounts, detection by use of radioactive tracers
++, detection possible without use of radioactive tracers
+++, present in large amounts.

cultures their metabolism is not affected by the presence of virus. Furthermore, Table 5 indicates that infected leaves and cultures contain comparable amounts of hydroxycinnamic acids and scopoletin: since TMV-infected cultures do not show any symptom of necrosis, there is also no direct relationship between the appearance of necrosis and levels of phenolics. This is also indicated by our preliminary experiments with hypersensitive tobacco leaves: accumulation of phenolics seems to occur after the formation of local lesions.

Our purpose is now to study the effect of TMV infection of hypersensitive plants on the individual enzymatic activities involved in the biosynthesis of scopoletin.

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A Comparative Study of Isoperoxidases of Tobacco as Influenced by TMV-Infection and Genetic Constitution

By

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In order to determine the variations in the spectrum of isoperoxidases of tobacco infected by TMV, the role of the genetic apparatus was studied by a comparative analysis of a hybrid and its parents at two ages. The experiments were carried out with the hybrid resulted from the crossing of two varieties of *Nicotiana tabacum*. The spectrum of isoenzymes was determined by zone electrophoresis on acrylamide gel, using leaf extracts at 11 days after germination in the 6—7 leaf stage. It was concluded that the virus acts on the genetic apparatus either by genetic repression-derepression mechanism, or by determining the synthesis of enzymatic proteins containing very weak S—S bonds. Both mechanisms could perhaps operate simultaneously and independently.

It is known that viral infections influence protein metabolism of the host, inducing also modifications in enzymatic activities. In the recent years, these type of studies became more intensive by investigating the isoenzyme composition of certain enzymes. Of these, peroxidases are perhaps the most thoroughly investigated group (FARKAS and STAHMANN, 1966, SHAPIRA (1967).

For the elucidation of the mechanisms by which the virus induces the disease symptoms, two problems must be solved: 1) whether new isoenzymes are specificaly produced in relation to infection, and 2) what is the nature of the new isoenzymes. In order to gain a deeper insight into these problems our aim was to investigate the dynamics of isoperoxidases in a hybrid of *Nicotiana tabacum* and its parents, as well as the action *in vitro* on the isoenzyme spectrum of some chemical agents which are known to determine modification of the structure of proteins.

Materials and Methods

The experiments were carried out with two varieties of *N. tabacum* (B. P. m. S. and Koro P. superelite) as well as with their hybrid. The analyses were made with 11-day-old leaves in the 6—7-leaf stage. Leaves were extracted with an acetate buffer at pH 4.8 containing 0.1 *M* potassium metabisulphide. For special extraction 6 *M* urea or 1 per cent sodium dodecylsulphate (SDS) was added to the buffer. A polyacrylamide gel prepared with tris-borate buffer, pH 8.0 was used

for the electrophoresis experiments. For special extraction 0.1 per cent urea or 0.1 per cent SDS was added to the gel. Electrophoretic studies were carried out at 4°C with a current of 220 V and 4 mA/tube. Samples of crude extracts were applied to the middle of the tubes using filter paper discs. We obtained, thereby, a more complete isoenzymatic spectrum containing both cathodic and anodic fractions.

Results and Discussion

It is seen in Fig. 1 that the isoenzyme composition is thoroughly modified after infection of the host with tobacco mosaic virus (TMV). The infected sample (hybrid) contains an extra anodic fraction and a cathodic one, as compared to the

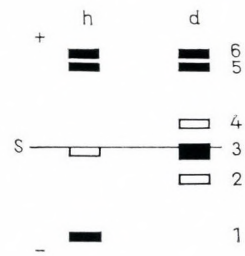

Fig. 1. Zymogram of peroxidases from healthy (h) and diseased (d) leaves in the 6—7-leaf stage. S=start

control (uninfected). One cathodic fraction, with a greater mobility, however, is lacking after infection. In order to learn more about the role of virus infection in inducing new bands of isoenzymes we investigated the changes in zymograms of the healthy and infected parents too. As is seen in Fig. 2 the healthy parents contain all the bands which the hybrid possesses except band 2 of the hybrid infected with TMV. The fact that band 4 is present in both parents as well as in the hybrid after infection, but not in the uninfected hybrid, can be attributed to a synthesis repression or to the inactivation of the existing enzyme.

Fig. 3 shows the results with the infected parents. It is seen that band 2 which appears in the infected hybrid, is also characteristic for both parents as a result of virus infection. In further experiments we investigated the action of SDS and urea on the isoenzyme production. Band 4 does not change as a result of extraction with SDS (Fig. 4). One can conclude from these results that the appearance of band 4 in the virus diseased hybrid is the result of a new enzymatic synthesis and it is not due to the activation of an inactive protein. As is known SDS does influence the tertiary structure of proteins, and still, band 4 is not changed.

As regards band 2, which appears as a consequence of the infection with TMV, it has to bear in mind that this fraction is not changed if the diseased tissue is extracted with SDS. However, it is identical, as far as its mobility is concerned,

with band 2 which appears in the healthy tissues under the influence of extraction with SDS. This means that its activity is not dependent on S–S linkages, and furthermore, that its genesis may be similar in virus-infected and in the SDS-treated tissues. Probably this isoenzyme is activated by breaking the S–S bridges in the inactive enzyme. Furthermore, one can suppose that it does not arise from

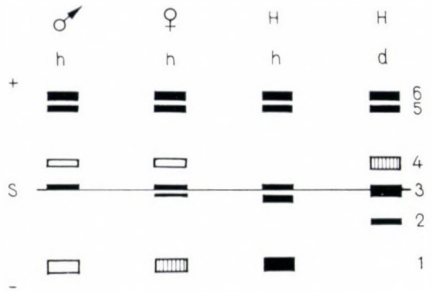

Fig. 2. Zymogram of peroxidases from healthy leaves of the hybrid (H), its parents (male and female) and diseased leaves (d) of the hybrid in the 6–7-leaf stage. S=start

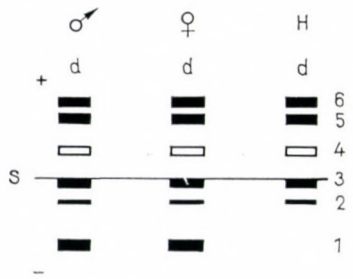

Fig. 3. Zymogram of peroxidases from diseases leaves (d) of the hybrid and its parents in the 6–7-leaf stage. S=start

fraction 1 in the healthy plants which have been extracted with SDS, and that band 1 disappears independently from band 2 (Fig. 5).

Under our experimental conditions urea, as an extracting agent, did not exert any influence upon isoperoxidases. The different behavior of the TMV-infected hybrid, as compared to the infected parents, could be explained by considering the specific ontogenetic isoperoxidasic dynamics of the healthy hybrid. In order to get more data on plants in an early ontogenetic stage (11 days after seed germination), we compared isoperoxidases in the hybrid as well as in the parents at this early ontogenetic stage. It is seen in Fig. 6 that bands 1, 4 and 6 are missing in the hybrid. The last two from these beeing likewise absent at this stage in the parents too. As regards band 1 virus infection induces an alteration in the hybrid in the 6–7-leaf stage which is similar to an early ontogenetic stage. An additional

event supporting this idea is the appearance of band 1 in the hybrid in this early stage of ontogenesis as a result of extraction with buffer containing urea. Under the influence of urea the H – H bridges break up activating thereby the isoenzyme which appears in the zymogram.

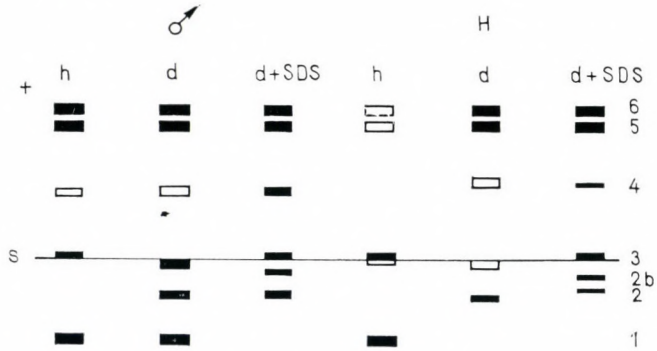

Fig. 4. Zymogram of peroxidases from the hybrid (H) and its male parent. h = healthy leaves, d = diseased leaves, d + SDS = diseased leaves extracted with SDS. 6–7-leaf stage. S = start

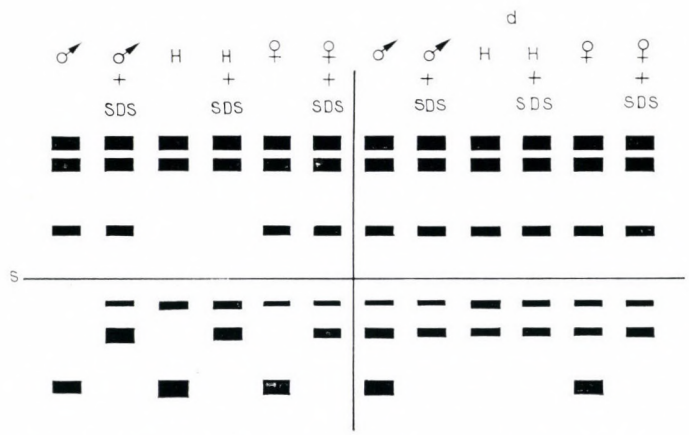

Fig. 5. Zymogram of peroxidases from healthy (h) and diseased (d) leaves of the hybrid (H) and its male and female parents. SDS = leaves extracted with SDS. 6–7-leaf stage. S = start

As a result of the experimental data mentioned above, one can conclude that the changes in the isoenzyme pattern in the virus-infected plant can be divided into two categories: Some isoenzymes exist in both the diseased hybrid or in the healthy parents (band 4). Others, however, do not exist in the healthy parents (band 2). As regards the first case, band 4, for example. can appear in the healthy hybrid in an ontogenetically more advanced stage, however, under the influence

of the disease, it appears earlier. In fact, this band remains unchanged after treatment with SDS or urea, an indication of a new synthesis by genetic derepression, and not a structural modification of an inactive protein. Bands belonging to the second category appear only as a consequence of infection by changing (activating) an inactive form, activating thereby the preexisting isoenzyme. These events can occur under the influence of unknown chemical factors similar in action to

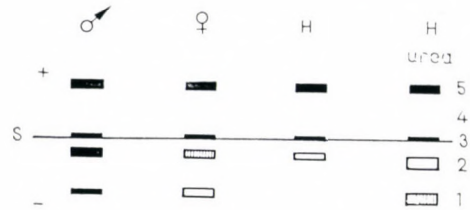

Fig. 6. Zymogram of peroxidases from healthy leaves of the hybrid (H) and its parents (male and female), urea = leaves extracted with urea. Age: 11 days. S = start

SDS or urea. These factors should be activated as a result of the diseased condition.

In conclusion, virus infection induces disturbances in the ontogenetic dynamics of isoenzymes, decoordinating the activity of some enzymes (EŞANU, 1969).

*

We wish to thank Prof. Dr. ANIELA KOZLOWSKA, Head of the Virus Laboratory of the Polish Academy of Sciences in Krakow, for providing seeds and Mr. F. PARAMON for the excellent technical assistance.

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Changes in Nucleolytic Enzymes in Virus-infected Tobacco Leaf Tissues

By

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An increase in the level of one of the nucleases in Xanthi tobacco upon local virus infection by TMV is supposedly due to an increase in abscisic acid in injured cells of local lesions.

Increase in the level of nuclease activity has been shown to occur in tobacco leaf tissues upon virus infection (DIENER, 1961). The tissues of higher plants are known to contain more than one nucleolytic enzyme (WYEN et al., 1971). The question arises as to whether virus infection affects the level of one enzyme only or the level of all nucleases is increased simultaneously. Progress in the isolation, purification and quantitative determination of higher plant nucleases (WYEN et al., 1969, 1971; UDVARDY et al., 1970) made it possible to answer this problem. The present report deals with the changes in the amount of nucleases present in Xanthi tobacco leaves after infection with tobacco mosaic virus (TMV).

Materials and Methods

Nicotiana tabacum cv. Xanthi plants were grown under ordinary greenhouse conditions. Half leaves were mechanically infected by TMV and after the development of local lesions (2 days after infection) the control and infected halves were extracted for a quantitative assay of the nucleases present.

The isolation, characterization and quantitative assay of the various nucleases weres carried out as described earlier (WYEN et al., 1969, 1971; UDVARDY et al., 1970).

Results and Discussion

The level of four major nucleolytic enzymes was measured: (a) nuclease I, an endoribonuclease which produces 2', 3'-cyclic phosphates as breakdown products, (b) nuclease II, a sugar non-specific endonuclease which produces 5'-nucleotides from both DNA and RNA, (c) an alkaline phosphodiesterase which breaks down both DNA and RNA by an exonucleolytic mode of action and produces 5'-nucleotides, and (d) an acid phosphodiesterase.

A summary of the results is given in Table 1. It may be seen that virus infection resulted in a significant increase in the level of nuclease I only.

Nuclease I has been shown in previous studies to be the enzyme in leaf tissues which responds to mechanical injury (WYEN et al., 1969, 1971.). Thus, it seems probable that it is mechanical injury which plays a major role in regulating the

Table 1

Level of nucleases in control and TMV-infected half leaves of Nicotiana tabacum cv. Xanthi

Enzyme	Activity Σ O. D./peak		% Activity in infected tissue (control = 100%)
	Control	Infected	
Nuclease I	312.0	655.2	210
Nuclease II	65.2	63.8	97
Phosphodiesterase (alkaline)	22.8	23.0	100
Phosphodisterase (acid)	154.3	166.0	107

level of nucleases in virus-infected leaves if the infection results in the formation of local lesions. Also, this is the nuclease the level of which is increased dramatically in leaf tissues treated with abscisic acid, and the level of which is depressed by kinetin (WYEN, ERDEI, UDVARDY, BAGI and FARKAS, unpublished results). On the other hand, nuclease II accumulates in leaf tissues upon normal ageing UDVARDY et al., 1970). Benzimidazole has a peculiar effect; it increases the level of nuclease I in short-term experiments and decreases it upon longer treatment.

To sum up the results show that the change in nuclease level upon virus infection (in local lesion combination) is due to an increase in the level of one nuclease only. On the basis of results obtained by treating leaf tissues with various plant growth regulators, and assaying the response of the level of individual nucleases, the hypothesis is advanced that abscisic acid produced in injured cells (local lesions) might be a factor responsible for triggering the changes in nuclease level.

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Nukleinsäure abbauende Enzyme in Blättern von *Triticum aestivum* nach Infektion mit *Puccinia graminis tritici*

Von

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Separations of extracts of healthy and rust-infected wheat leaves by column chromatography on hydroxyapatite demonstrated 10 different RNase fractions. The activity of three fractions was increased following infection. Most conspicuous was the increase of fraction I. At the 5th dpi it had reached 3 times, at the 9th dpi 10—15 times the specific activity of the corresponding fractions obtained from healthy leaves. In extracts of noninfected and infected leaves 5 DNases were demonstrated, all of which were increased after infection. PDase was detected in 3 fractions with no difference in activity between healthy and infected leaves.

Im Zusammenhang mit dem unter dem Einfluß des Parasiten stark stimulierten RNS-Stoffwechsel im rost-infizierten Blatt (vgl. HEITEFUSS, 1966) muß auch den Veränderungen der RNase*-Aktivität Beachtung geschenkt werden.

Eine Aktivitätserhöhung dieses Enzyms in rost-infizierten Weizen- bzw. Bohnenblättern wurde von ROHRINGER et al. (1961), TSCHEN (1966), FRIČ und FUCHS (1970) und SACHSE und WOLF (1970) festgestellt. Darüberhinaus konnte auch für die im Falle von Wirt-Parasit-Kombinationen bisher nicht beachtete DNase** eine stark erhöhte Aktivität, für die PDase*** jedoch keine Beeinflussung nachgewiesen werden.

Diese Untersuchungen wurden ausschließlich mit Rohextrakten durchgeführt. Es ist jedoch bekannt, daß in pflanzlichen Geweben mehrere, verschiedene RNasen (vgl. BARNARD, 1969) und, wie in neuerer Zeit gezeigt, auch verschiedene RNase-Isozyme (WOLF, 1968, RANDLES, 1968, WILSON, 1969) und PDase-Isozyme (LERCH, 1968, WOLF, 1968) vorkommen.

Für eine differenzierte Analyse der durch den Parasiten verursachten Veränderungen der Enzymaktivitäten schien uns daher eine möglichst weitgehende Auftrennung der Nukleasen erforderlich zu sein.

* = Ribonuklease
** = Desoxyribonuklease
*** = Phosphodiesterase

Das Ziel der vorliegenden Arbeit war, zu prüfen, inwieweit nach säulenchromatographischer Auftrennung von Rohextrakten nichtinfizierter und infizierter Weizenblätter qualitative und/oder quantitative Unterschiede im Spektrum der RNasen, DNasen und PDasen bestehen.

Material und Methoden

Anzucht, Inokulation und Extraktion der Pflanzen erfolgten wie bei SACHSE und WOLF (1970) angegeben. Von den Rohextrakten wurde eine jeweils 25 mg Protein enthaltende Flüssigkeitsmenge auf 1.5 cm × 20 cm Säulen aus Hydroxylapatit (hergestellt nach LEVIN, 1962) aufgetragen. Die Elution erfolgte mit Phosphatpuffer p_H 6.8, dessen Konzentration von 0.005–0.3 M linear anstieg. In jedem zweiten der 4–6 ml Flüssigkeit enthaltenden Röhrchen wurden RNase, DNase und PDase in der bei SACHSE und WOLF (1970) beschriebenen Weise bestimmt.

Wegen der relativ geringen Enzymkonzentration im Eluat mußte die Inkubationszeit für RNase auf $2^1/_2$ Stdn., für DNase auf 24 Stdn. und für PDase auf 1 Std. (bei jeweils 45 °C) verlängert werden.

Bei einer gleichzeitigen Erfassung von RNase, DNase und PDase war es jedoch nicht möglich, einen Puffer-Gradienten zu ermitteln, der zur vollständigen Abtrennung der einzelnen Fraktionen führte. Wie die Abbildungen 1–4 bzw. 6–11 zeigen, war es bei guter Reproduzierbarkeit möglich, die einzelnen "peaks" eindeutig zu identifizieren.

Für die Bestimmung der p_H-Optima der RNasen wurde ein 0.1 *M* Citronensäure-Citrat-Puffer verwendet.

Ergebnisse

Sowohl für nichtinfizierte als auch infizierte Blätter konnten nach Auftrennung insgesamt 10 verschiedene Fraktionen mit *RNase*-Aktivität nachgewiesen werden (Abb. 1, 2, 3 und 4). Qualitativ neue RNasen, die entweder pilzlicher oder pflanzlicher Herkunft sind, traten nicht auf. Dagegen waren deutliche quantitative Unterschiede im Spektrum der RNasen zu beobachten.

Ein Vergleich von Trennungen nichtinfizierter und infizierter Blätter vom 5. dpi und 9. dpi zeigt eine deutliche Erhöhung der RNase-Aktivität der I., III. und V. Fraktion infizierter Blätter (Abb. 1, 2, 3 und 4). Geringfügige Unterschiede der übrigen Fraktionen konnten bei 6 unabhängig voneinander durchgeführten Versuchen nicht gesichert werden. Die mit der Front laufende I. Fraktion ließ sich von der folgenden gut abtrennen, so daß in diesem Fall eine Berechnung der spezifischen Aktivität möglich war. Sie betrug bei infizierten Blättern am 5. dpi das 2–3fache, am 9. dpi das 10–15fache derjenigen nichtinfizierter Blätter

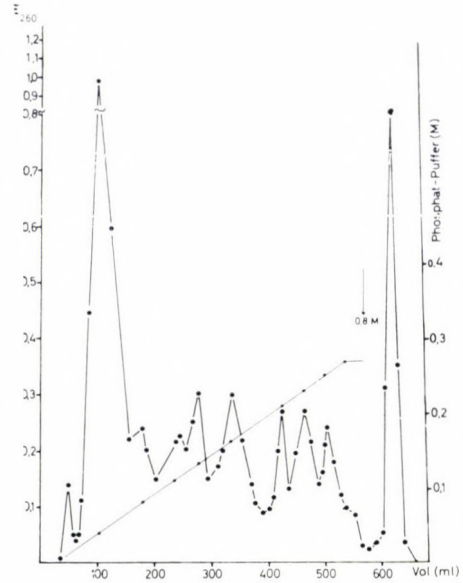
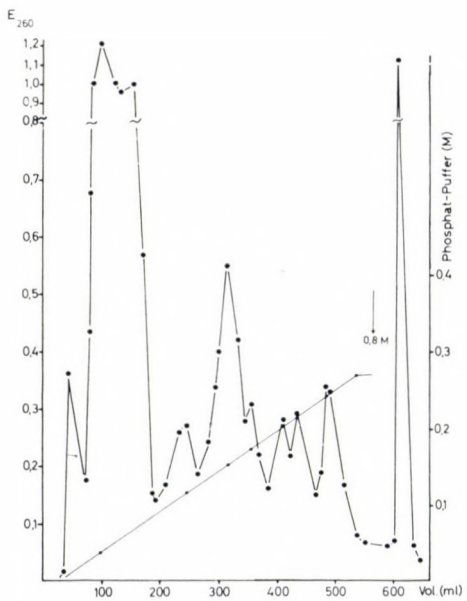

E 260
1,2
1,1
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,8 M
Phosphat-Puffer (M)
0,4
0,3
0,2
0,1
100
200
300
400
500
600
Vol. (ml)

Abb. 1


E 260
1,2
1,1
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0,4
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0,1
0,8 M
Phosphat-Puffer (M)
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600
Vol. (ml)

Abb. 2

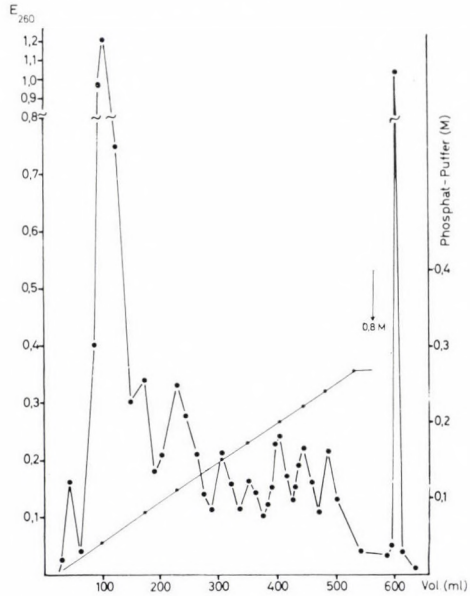

Abb. 3

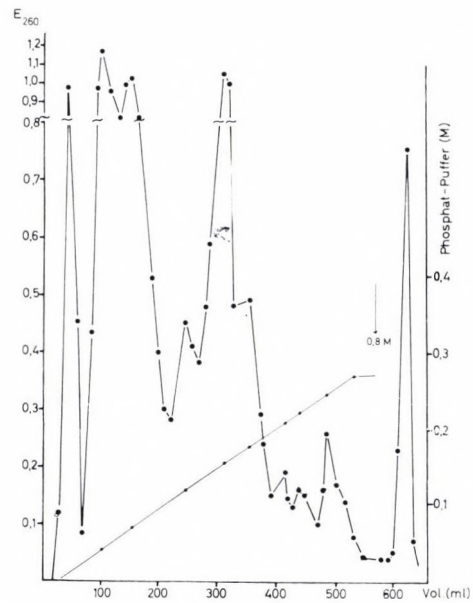

Abb. 4

Abb. 1—4. Chromatographische Auftrennung der RNasen aus Extrakten nichtinfizierter Blätter (Abb. 1 [5.dpi] und Abb. 3 [9. dpi]) und infizierter Blätter (Abb. 2 [5. dpi] und Abb. 4 [9. dpi])

Auffällig war weiterhin, daß von den nach Infektion stark erhöhten Fraktionen die III. Fraktion ihren maximalen Wert bereits am 5. dpi, die I. und V. Fraktion dagegen erst am 9. dpi erreichten. Eine ähnliche Beeinflussung einer Wirts-RNase aus Turnip yellow mosaic Virusinfizierten Chinakohlblättern beobachtete RANDLES (1968). Die in älteren Blättern kaum nachweisbare RNase zeigte nach systemischer Infektion eine starke Erhöhung ihrer Aktivität.

Die Proteine der letzten Fraktion (10.) waren relativ fest an den Austauscher adsorbiert und konnten daher nur mit einem 0.8 *M* Phosphatpuffer eluiert werden

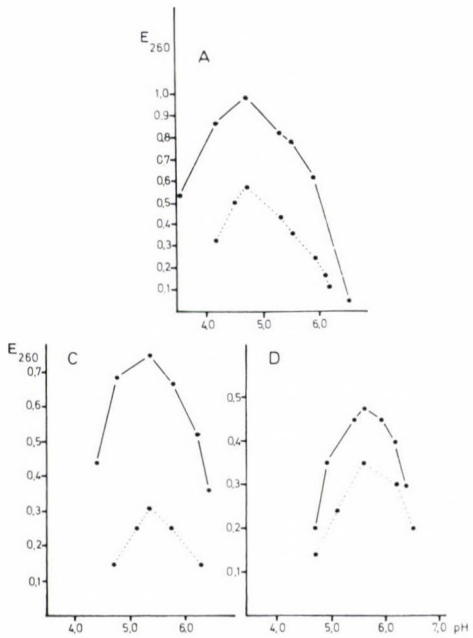

Abb. 5. Abhängigkeit der RNase-Aktivität vom p_H-Wert. Kurve A = 1. Fraktion; Kurve C = 3. Fraktion; Kurve D = 5. Fraktion; nichtinfiziert ●·····● ; infiziert ●——●

Eine weitere Auftrennung mit einem Puffer mit linear ansteigender Konzentration von 0.3 – 0.8 *M* war nicht möglich, sodaß angenommen werden kann, daß es sich hierbei um eine einheitliche Fraktion handelt.

Zur weiteren Charakterisierung der nach Infektion in ihrer Aktivität stark erhöhten Fraktionen I, III und V wurden die p_H-Optima und der Einfluß verschiedener Ionen bzw. Substanzen untersucht. Hierbei ergaben sich für die entsprechenden Fraktionen nichtinfizierter und infizierter Blätter jeweils gut übereinstimmende p_H-Optima. Unterschiede wurden jedoch zwischen einzelnen Fraktionen festgestellt. Während Fraktion III und V ein Optimum von 5.4 bzw. 5.6 in dem für pflanzliche RNasen typischen Bereich haben (vgl. BARNARD, 1969), wurde für die Fraktion I ein p_H-Optimum von 4.7 ermittelt (Abb. 5).

Tabelle 1

	Konzentration im Testansatz *M*	Verbleibende Aktivität (%)					
		I. Fraktion		III. Fraktion		V. Fraktion	
		nichtinfiz.	infiz.	nicht-infiz.	infiz.	nicht-infiz.	infiz.
keine	—	100	100	100	100	100	100
$HgCl_2$	10^{-3}	20—30	20—30	25	25	20	20
NaCl	10^{-2}	80—90	85	90	90	85	90
$CuSO_4$	10^{-3}	100	100	100	100	100	100
EDTA	10^{-3}	96	94	95	93	100	100
Jodacetat	10^{-3}	103—112	111	100	100	100	100

Die Enzymaktivität der einzelnen Fraktionen wird durch verschiedene Ionen bzw. Substanzen in ähnlicher Weise beeinflußt (Tab. 1).

Die RNase-Aktivität aller 3 Fraktionen wurde durch $HgCl_2$ stark gehemmt, während NaCl eine geringfügige Erniedrigung bewirkte. Unter dem Einfluß von EDTA war die Aktivität der I. und III. Fraktion leicht gehemmt; Jodacetat dagegen bewirkte eine leichte Aktivierung des Enzyms der I. Fraktion.

Ebenso wie bei RNasen konnten auch für *DNasen* nach Auftrennung der Rohextrakte infizierter Blätter keine qualitativen Unterschiede zu nichtinfizierten Blättern festgestellt werden. Von den insgesamt 5 verschiedenen Fraktionen waren nach Infektion alle erhöht (Abb. 6, 7, 8 und 9). Der größte Teil der gesamten DNa-

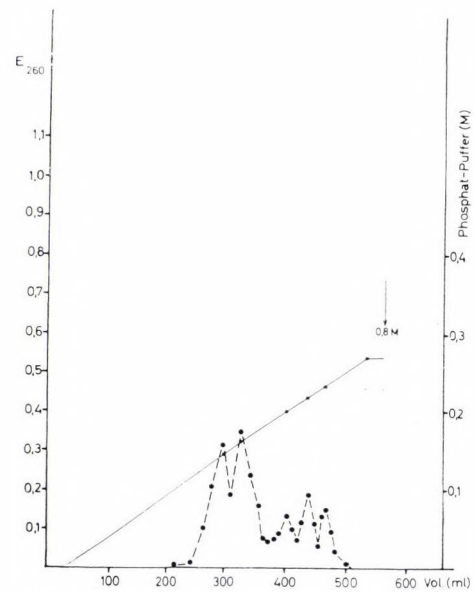

Abb. 6

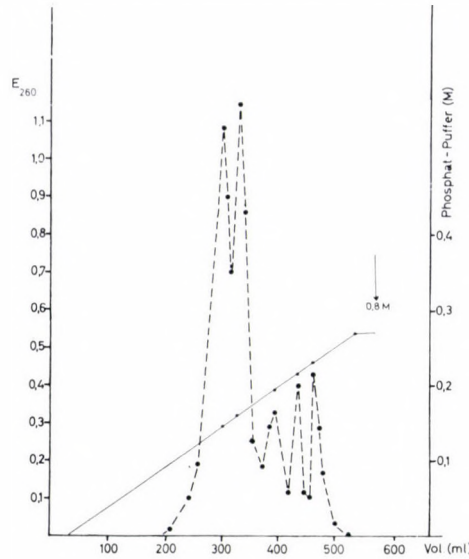

Abb. 7

se-Aktivität entfiel am 5. dpi auf die ersten beiden DNase-Fraktionen (Abb. 7), deren Aktivität bis zum 9. dpi wieder abfiel, während gleichzeitig die Aktivität der 3 übrigen Fraktionen zunahm, so daß diese nun etwa die Hälfte der Gesamt-Aktivität ausmachten.

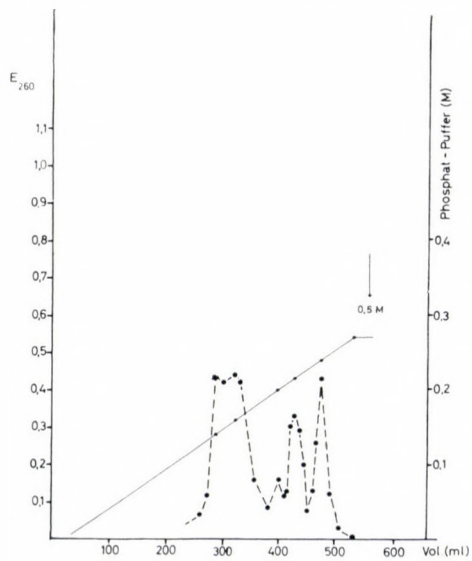

Abb. 8

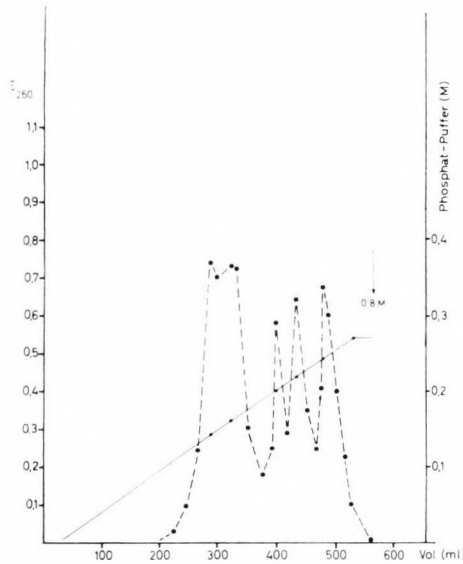

Abb. 9

Abb. 6—9. Chromatographische Auftrennung der DNasen aus Extrakten nichtinfizierter Blätter (Abb. 6 [5. dpi] und Abb. 8 [9. dpi]) und infizierter Blätter (Abb. 7 [5. dpi] und Abb. 9 [9. dpi])

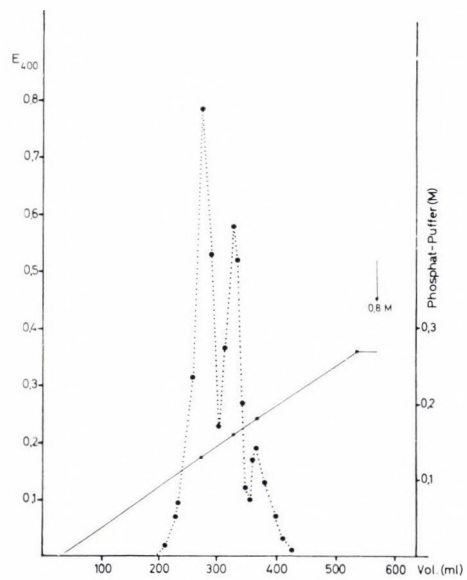

Abb. 10

Für die *PDase* wurden 3 verschiedene Fraktionen nachgewiesen (Abb. 10 und 11). Es ließen sich weder qualitative noch quantitative Unterschiede zwischen nichtinfizierten und infizierten Blättern feststellen, wie dies auch auf Grund der mit Rohextrakten gewonnenen Ergebnisse zu erwarten war. Den größten Anteil an der gesamten PDase-Aktivität hatten sowohl in nichtinfizierten wie auch in infizierten Blättern die ersten beiden Fraktionen.

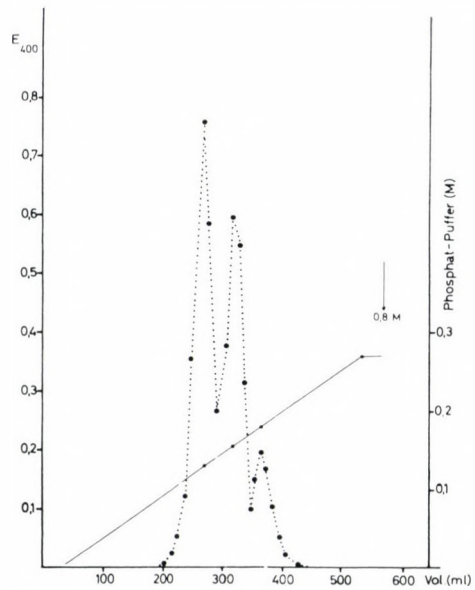

Abb. 11

Abb. 10—11. Chromatographische Auftrennung der PDase aus Extrakten nichtinfizierter Blätter (Abb. 10 [5. dpi] und infizierter Blätter (Abb. 11 [5. dpi])

Ebenfalls 3 PDase-Fraktionen konnte WOLF (1968) mit Hilfe der Disk-Elektrophorese in Polyacrylamid-Gelen nachweisen. Diese dürften mit den nach Hydroxylapatit-Chromatographie erhaltenen Fraktionen identisch sein.

Diskussion

Die in Rohextrakten festgestellte Erhöhung der RNase- und DNase-Aktivität (SACHSE und WOLF, 1970) wird durch die Ergebnisse der vorliegenden Arbeit bestätigt. Auf Grund des gleichen Enzymspektrums nicht infizierter und infizierter Blätter kann angenommen werden, daß von der Aktivitätserhöhung sowohl der RNase wie auch der DNase die Enzyme des Wirts betroffen sind. Inwieweit es sich dabei um eine Enzymneusynthese handelt, die auf eine vom Parasiten be-

wirkte Derepression zurückgeführt werden könnte, oder um eine Freisetzung latenter, membrangebundener Enzyme, muß durch weitere Versuche geklärt werden.

RNase-Erhöhungen wurden nicht nur nach Infektion pflanzlicher Gewebe mit Pilzen (ROHRINGER et al., 1961; FRIČ u. FUCHS, 1970; TSCHEN, 1966) und Viren (REDDI, 1959); DIENER, 1961; RANDLES, 1968), sondern darüberhinaus auch in mechanisch verletzten Blättern nachgewiesen (vergl. UDVARDY et al., 1969). Inwieweit die RNase-Erhöhung rost-infizierter Blätter mit einer allgemein gültigen Reaktion der Pflanze auf »stress«-Bedingungen erklärt werden kann (vergl. FARKAS et al. 1964) oder auf spezifischen Vorgängen im Zusammenhang mit dem Nukleinsäurestoffwechsel beruht, soll durch weitere Versuche geprüft werden.

Über die Eigenschaften der einzelnen Nukleasen läßt sich an Hand der bisher vorliegenden Daten wenig aussagen. Ein Vergleich des RNase- und DNase-Eluogramms zeigt, daß es sich bei den RNase-Fraktionen I–III und X um spezifische Ribonukleasen handelt; die Fraktionen IV–IX könnten unspezifische Nukleasen sein.

Die verschiedenen p_H Optima der 3 nach Infektion stark erhöhten RNasen deuten auf Enzyme mit unterschiedlichen Eigenschaften hin. Dies wird durch die Ergebnisse einer disk-elektrophoretischen Trennung dieser Fraktionen unterstrichen. (SACHSE und WOLF, unveröffentlicht). Sie ergab für die Fraktion I zwei, für die Fraktion III vier und für die Fraktion V eine Bande(n). Unterschiede zwischen nicht infizierten und infizierten Blättern konnten auch in diesem Fall nicht festgestellt werden.

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Ethylene Production, Tissue Senescence and Local Virus Infections

By

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Ethylene promotes the expression of one of the most common symptoms of ageing: the suppression of protein and nucleic acid synthesis. Artificial inhibition or promotion of protein synthesis results in an increased or decreased evolution of ethylene, respectively. In hosts with viral local lesions caused by TMV and TNV, a certain degree of enhanced ethylene production was demonstrated before the appearance of local lesions. However, the mass of the evolution of ethylene occurred at the very beginning of tissue necrosis. In systemic infections the rate of ethylene production remained unchanged. In another local virus infection (bean yellow mosaic virus on *Tetragonia expansa*), causing chlorotic spots instead of necroses, the evolution of ethylene also increased. It is concluded that the increased production of ethylene is connected to local tissue senescence caused by local virus infections but not necessarily to local necroses.

Our previous experiments (BALÁZS, GÁBORJÁNYI, TÓTH and KIRÁLY, 1969) have shown that the production of ethylene – a hormone of plant senescence – markedly increases as a result of virus infection in a local lesion host. This finding has been supported by similar results of NAKAGAKI, HIRAI and STAHMANN (1970). The increased production of ethylene is associated with tissue necrosis (local lesion production) induced by virus infections. This is the conclusion of our experiments mentioned above in which we infected a single cultivar of tobacco (namely Xanthi-nc) with two viruses, one of which induces local lesions in infected leaves (tobacco mosaic virus) and another which spreads systemically in the tissues without tissue necrosis (cucumber mosaic virus). Ethylene increases *only* if the host is infected with tobacco mosaic virus (TMV), however, ethylene production does not change in the *same cultivar* if it serves as a systemic host for cucumber mosaic virus (CMV). This finding proved to be valid also for another local virus infection, associated with tissue necrosis: in recent experiments we provided evidence for increased ethylene production in Pinto bean leaves infected with tobacco necrosis virus (TNV).

Role of Ethylene in Plant Senescence

As regards local lesions (tissue necroses) induced by viruses, our previous experience has shown that local lesion development is promoted if tissue senescence is enhanced in the host by different means (KIRÁLY, EL HAMMADY and

POZSÁR, 1968). On the other hand, local lesions are suppressed if juvenility is promoted in the host by applying cytokinin-type hormones or by removing the terminal bud. Additional support for this idea came from the experiments of OPEL (1965) and recently by NAKAGAKI and MATSUI (1971). All these findings show that the development of local lesions (necroses) is somehow connected to the ageing of host tissues.

Table 1

The effect of ethylene on the incorporations into the TCA insoluble fraction of Pinto bean leaves (cpm/g fr. wt.)

	Control	Ethylene 10^3 ppm
Methionine-C^{14}	3505	465
Orotic acid-C^{14}	445	180

Table 2

The effect of treatments promoting or suppressing protein synthesis on the ethylene production in Pinto bean plants

Treatment	Ethylene (μl/g) day	Per cent
Control	0.78	100
Chloramphenicol (200 ppm)	1.13	145.0
Fluorophenylalanine (100 ppm)	1.58	202.5
Puromycin (50 ppm)	1.11	142.0
Cycloheximide (10 ppm)	0.72	92.0
Heat treatment (50°C, 20 sec.)	1.15	275.5
Benzyladenine (30 ppm)	0.42	58.6
Decapitation	0.49	63.1

The phytogerontological effect of ethylene was proposed by HALLAWAY and OSBORNE (1969) and, recently, undoubtedly demonstrated by ABELES, CRAKER and LEATHER (1971). Accordingly, these workers believe that ethylene is required for the process of ageing. Similarly, we were able to show that ethylene markedly inhibits protein and nucleic acid synthesis in leaves. This result is summarized in Table 1, showing an inhibition of incorporation of precursors of protein and RNA into the TCA-insoluble fraction of Pinto bean leaves. Thus, ethylene seems to promote the expression of one of the most common symptoms of ageing: the suppression of protein and nucleic acid synthesis.

It is of interest that artificial inhibition of protein synthesis results in the yellowing of leaf tissues. This is a typical senescence effect, which is in connection with increased ethylene production (Table 2). On the other hand, both ethylene

and ageing are suppressed by treatments causing juvenility in plants (decapitation or treatment with the cytokinin benzyladenine). This is the conclusion of experiments summarized in Table 2. Primary leaves of Pinto bean plants were brushed daily for 5 days with antibiotics inhibiting protein synthesis or with benzyladenine which stimulates the syntheses in plants causing, thereby, a juvenility effect. Decapitation was made at the appearance of the first trifoliate leaf. Heat treatment was performed by immersing primary leaves of Pinto bean in hot water (50°C) for 20 min. Ethylene production was measured gas-chromatographically as described earlier (BALÁZS, GÁBORJÁNYI, TÓTH and KIRÁLY, 1969). The results indicate that protein inhibitors promote the production of ethylene as well as the process of ageing. One apparent exception is cycloheximide. This antibiotic although inhibiting protein synthesis does not induce ethylene production. Senescence (yellowing) of leaves does not occur in this case, which may be a reasonable consequence of the unchanged level of ethylene. As is seen, the promotion of juvenility suppress ethylene as well as ageing in the leaves. Consequently, the data presented here suggest that ethylene is connected to plant senescence. As regards the origin of ethylene, this is still a disputed question.

Very recently, DEMOREST and STAHMANN (1971) proposed a model for the production of ethylene in plants from peptides and protein containing methionine. According to their suggestion proteolysis in plants may increase ethylene production by producing peptides with a C-terminal methionine residue. Peptides of this type produce ethylene in a peroxidase system at significant rates. It has been reported that oxidases are activated in virus-infected plants (FARKAS, KIRÁLY and SOLYMOSY, 1960, VAN LOON, 1971, see the first paper in this volume, pp. 9—20). However, an increase in proteolytic activity during local virus infections has not been observed as yet, although one can suppose that local yellowing or local senescence is connected with a certain degree of proteolysis. This remains to be demonstrated in further experiments.

Role of Ethylene in Local Lesion Production

We supposed that local necrotic spots induced by TMV could be the consequence of an enhanced local senescence in host leaf tissues caused or followed by ethylene production. Supporting the first possibility it would be the demonstration of an enhanced production of ethylene *before* the appearance of local lesions. Accordingly, we measured ethylene production in Xanthi tobacco leaves infected with TMV in 3 hr intervals. We infected a series of uniform plants in the greenhouse and samples have been taken at 21, 24, 27, 30 and 33 hr after infection for determining the evolution of ethylene. Experimental methods of measuring ethylene production were described earlier (BALÁZS, GÁBORJÁNYI, TÓTH, and KIRÁLY, 1969). As is seen in Fig. 1 ethylene production increased at the very beginning of tissue necrosis in the case of local lesion infection (TMV). A certain degree of enhanced

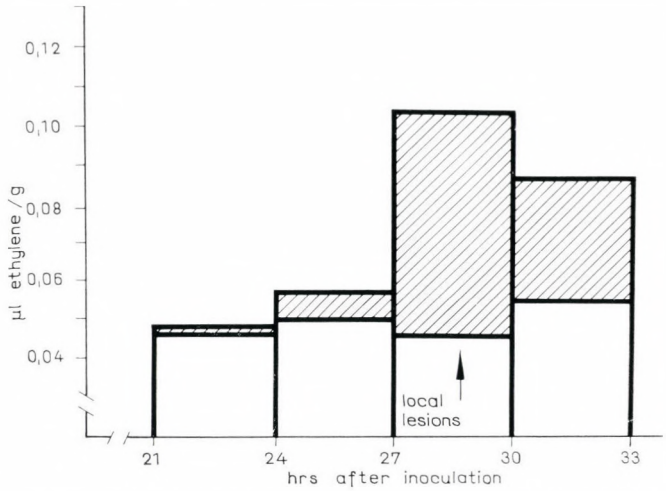

Fig. 1. Ethylene production in Xanthi tobacco leaves infected with TMV. Open bars: uninfected, solid bars: infected

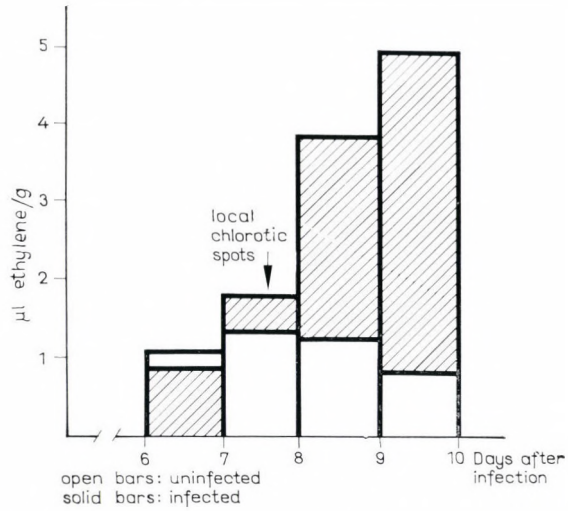

Fig. 2. Evolution of ethylene in *Tetragonia expansa* infected with bean yellow mosaic virus. Open bars: uninfected, solid bars: infected

ethylene production was demonstrated before the appearance of local lesions. However, this enhancement is not enough convincing. An impressive increase in the evolution of ethylene occurs somewhat later: at the beginning of lesion development. Thus, our endeavour to demonstrate a cause-and-effect relationship of ethylene with local senescence or local necrosis resulted in uncertain conclusion.

It still remained the possibility that the increased ethylene production perhaps was the consequence and not the cause of tissue decomposition (necrosis).

To gain a deeper insight into this mechanism we investigated the ethylene production in the case of another local virus infection causing chlorotic spots instead of necrosis. In the host-parasite combination of *Tetragonia expansa* and bean yellow mosaic virus the chlorotic spots of infected leaves represent an advanced degree of tissue senescence, and still, without tissue necrosis (without local lesions). As is seen in Fig. 2 ethylene production was increased also in this host-parasite combination at the time of the appearance of chlorotic local spots. On the basis of this result one can conclude that the increased ethylene production of the local lesion hosts is not the consequence of the development of tissue necrosis.

In summary, on the basis of our experimental data it is not possible to postulate unequivocally that viral lesions are the results of an increased ethylene production. On the other hand, it is also true that viral lesions are not causing an increase in the production of ethylene. We believe that the experiments described here present evidence for the association of local virus infections with local senescence and with an increased evolution of ethylene.

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Some Properties and Functions of the Coat Protein of Plant Viruses, Including the Function of Host-range Control

By

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The polyfunctionality of the structural proteins of different viruses is discussed in this paper. Some data is presented concerning the role of plant virus coat protein in the first stages of infection (in the function of host range control) and in the phenomenon of the recognition at the "protein-RNA" and the "protein-protein" levels (morphopoetic function of the structural protein).

It is well known that the structural protein of some simple viruses can perform different functions in relation to the virus-cell interaction. The protective functon is the most obvious, though not the only, function of the coat protein. It can also play an important role in controlling the host range of different animal viruses and bacteriophages. The coat protein molecules do participate in the morphopoesis of the virion, hence their morphopoetic function. The coat protein of RNA-phages and, perhaps, of some other viruses plays a role — as a repressor — acting at the translation level. The coat protein of f2 phage is also capable of inducing lysis of bacterial cells and the coat protein of ϕX 174 phage probably participates in the replication of the replicative form of its DNA. Thus, the polyfunctionality of the viral coat protein is rather useful ensuring realization of several different functions with minimum genetic information.

The Role of the Structural Protein of Plant Viruses in the Host Range Control

In our experiments studying the role of the structural proteins we applied two possibilities in approaching the problem. According the first one we investigated the interference between the plant virus and the homologous viral protein. In these experiments we used *Chenopodium amaranticolor* as a host which is universally susceptible to a great number (about one hundred) of different viruses.

It is obvious that the information obtained in experiments on the interference is indirect, since the specificity of interference between the virus and the homologous viral protein was equated with the specificity of interaction between the virus particles and cell receptors.

It was shown that viral proteins of barley stripe mosaic virus (BSMV) or tobacco mosaic virus (TMV) interfere with the homologous viruses when added prior to or simultaneously with the virus. Proteins from the foreign viruses do not inhibit virus infectivity. Interference did not occur between free viral RNA and the homologous viral protein.

Because the inhibitory effect was a specific one, in other words it was produced only by the homologous but not by the foreign viral protein, one could suggest that it reflected to the specific competition between the virus and viral protein for the cell receptors. These results suggest also that a set of different receptors exists in *C. amaranticolor* some members of which are specific to a certain virus but not to other viurses. For example, receptors specific for TMV do not absorb BSMV or even CV4 (cucumber virus 4).

These data permit to make a conclusion as follows: The virus must be adsorbed to the host before uncoating of its RNA takes place. If so, the structural protein of a plant virus plays a specific role in the host range control.

In further experiments we used hybrid viruses reconstituted *in vitro* for studying this question. The results are summerized in Fig. 1. The general scheme of the experiment is seen in Fig. 1A. Here the viruses designated as *RNA PROT* or *rna prot* are infective for P' and P'', respectively. Plant designated as P''' is a common host, being susceptible to both viruses. Using different combinations of protein and RNA from the two viruses one can reconstitute the hybrid viruses of different composition to study their host range (see Fig. 1A).

It was shown that hybrid virus consisting of TMV-protein and BMV–RNA (bromegrass mosaic virus-RNA) was not able to infect the corn and barley plants, susceptible to BMV (Fig. 1B). On the other hand, free RNA isolated from hybrid virus was infective to barley plants. It is of importance that this hybrid virus was infective to *Chenopodium quinoa* which is a host common for both TMV and BMV.

The observation that ($BMV_{RNA} \times TMV_{protein}$) hybrid is unable to infect the barley and corn may have different explanations, one of which is that TMV coat protein is nonfunctional in these cells.

It is of interest that the hybrids consisting of CV4 protein and RNA from TMV or PVX (potato virus X) were infective to some plants resistant to CV4 (Fig. 1, C, D).

These observations show that the coat protein of a foreign virus normally unable to attack a host do not necessarily change the host range of heterologous viral RNA enclosed in hybrid particles.

These results are seemingly in contradiction with our suggestion mentioned above, that the first stages of infection (at least adsorption) are specific in virus-host interaction. One can think that the mechanisms of the host range control are not operative in the first stages of infection in the case with ($CV4_{protein} \times TMV_{RNA}$) and ($CV4_{protein} \times PVX_{RNA}$) hybrids as they were infective to *Nicotiana glutinosa* (see Fig. 1, C and D). However, this contradiction can be explained, at

least in the case studied. It was shown that ($CV4_{protein}$ × TMV_{RNA}) hybrid, infective to *C. amaranticolor* in spite of containing CV4 protein, was specifically inhibited with CV4 protein but not with TMV protein. Hence one can suggest that *C. amaranticolor* cells do contain receptors specific for CV4 which bind with

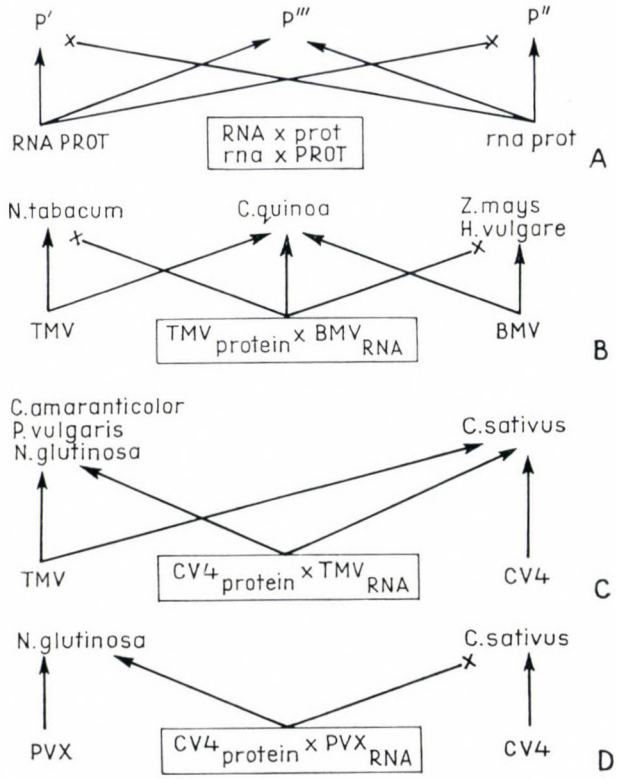

Fig. 1. The marks → and —× stand for the infectivity and non-infectivity of the virus to the appropriate host

CV4 protein but not with TMV protein and adsorb the hybrid particles ($CV4_{protein}$ × TMV_{RNA}). In fact, the observation that this hybrid virus is able to infect *C. amaranticolor* does not mean at all that the host range-controlling mechanism does not operate at the first stages in this case. On the contrary, we can suggest that *C. amaranticolor* possesses cell receptors specific for CV4 in spite of being nonsusceptible to this virus. Therefore, the structural protein of plant virus would perform the function of host range control even in this case. It seems reasonable to suggest that a set of receptors which the plant cell possesses, sometimes includes the receptors to a certain virus(es) unable to infect this cell.

Morphopoetic Functions of the Structural Protein

It is well known that the low-molecular weight protein of some viruses can be repolymerized *in vitro* into protein shells, structurally similar to intact virus. On the other hand, the infective virus can be reconstituted from RNA and protein under certain conditions, unfavorable for the protein repolymerization. Therefore, two different phenomena can be defined on which the morphopoetic function of the coat protein is based: recognition on the level of a) "protein-protein" and b) "protein-RNA".

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Some Peculiarities of Protein Synthesis in Infected Plant Tissues

By

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Biochemical effects of a facultative *(Botrytis cinerea)* and an obligate parasite *(Erysiphe cichoracearum)* on host protein synthesis is compared. In the resistant combinations of hosts and pathogens the contents of proteins as well as ribosomes increased more intensively than in the susceptible ones. The incorporation of labelled leucine by ribosomes is also stimulated in the resistant combinations. Qualitative and quantitative changes in ribosomal activity upon infection were also demonstrated. It is concluded that the proteins which are synthesized in the resistant combinations may play a role in disease resistance.

Investigations on the biochemistry and physiology of infected plants demonstrated significant alterations in the protein of the host tissues (RUBIN and ARTSIHOVSKAYA, 1968, STAHMANN, 1965, LIPSITS, 1970). However, the effect of infection on the properties of protein-synthesizing system in plant tissues is practically unknown. Some observations have been published on the formation of ribosomes in infected plant tissues (AKAZAVA 1956, HEITEFUSS, 1968).

This report presents the results of investigations on some properties of ribosomes in healthy and diseased plant tissues. The effect of a facultative parasite *Botrytis cinerea* on stored cabbage and the effect of an obligated parasite, *Erysiphe cichoracearum* on a cucumber was studied.

It is known that synthesis of proteins in host tissues is a common reaction of plants to infection (STAHMANN, 1965, RUBIN, IVANOVA and DAVIDOVA, 1965, FARKAS and STAHMANN, 1966). In our experiments, we also observed an increase of protein content in cabbage tissues upon infection by *B. cinerea* (Table 1). In the resistant variety Amager, protein synthesis was more intensive as compared to the susceptible variety Nomer Pervi. The question is, which pathways are involved in the increase of protein content in infected tissues. One possibility would be an alteration in the content of ribosomes, the amount of which can determine the total intensity of protein synthesis in the cell. Indeed, in the infected tissues of cabbage the amount of ribosomes increases, and more significantly, it does increase to a higher extent in the resistant variety (Table 2).

Another explanation for an increase in protein content in infected tissues would be an increase in ribosome activity (Table 3).

As indicated in Table 3, infection stimulated the capacity of ribosomes to incorporate C^{14}-leucine into protein. Incorporation of the labelled precursor into proteins in resistant variety increased to a greater extent than into proteins of the

Table 1

The effect of infections on the protein content in plant tissues (mg/g fresh weight)

Host-parasite combination	Variety	Tissue	Protein	%
Cabbage—*Botrytis cinerea*	Resistant Amager	Healthy	3.43±0.11	100
		Infected	4.89±0.31	143
	Susceptible Nomer pervi	Healthy	2.38±0.06	100
		Infected	2.82±0.06	118
Cucumber—*Erysiphe cichoracearum*	Resistant Dalnevostochni	Healthy	6.8±0.3	100
		Infected	8.9±0.22	131
	Susceptible Nerosimi	Healthy	9.7±0.85	100
		Infected	9.3±0.69	96

Table 2

Content of ribosomes in plant tissues infected by parasites (μg/g fresh weight)

Host-parasite combination	Variety	Tissue	Content rib RNA	%
Cabbage—*Botrytis cinerea*	Resistant Amager	Healthy	46.5±1.96	100
		Infected	64.0±5.71	140
	Susceptible Nomer pervi	Healthy	34.0±1.70	100
		Infected	39.8±1.93	115
Cucumber—*Erysiphe cichoracearum*	Resistant Dalnevostochni	Healthy	85.2±6	100
		Infected	111.0±15	130
	Susceptible Nerosimi	Healthy	228.1±10	100
		Infected	176.0±9	76

susceptible one. The increase of ribosomal activity under the influence of *B. cinerea* seems to be the result of alterations in the properties of this fraction and is probably not connected with "preribosomal" steps of protein synthesis. On combination of the ribosomes from healthy tissues with supernatant proteins from infected tissues, the ribosomal activity was suppressed (Table 4). A non-compati-

bility of ribosomes from healthy tissues and proteins from the supernatant of the infected tissues was observed.

In the case of the opposite combination the non-compatibility was less significant. The reason of this phenomenon is unclear and requires further investi-

Table 3

C^{14}-leucine incorporation by ribosomes in a cell-free system (counts/100 sec/1 mg RNA)

Host-parasite combination	Variety	Tissue	Incorporation	%
Cabbage—*Botrytis cinerea*	Resistant Amager	Healthy	1235±62	100
		Infected	1730±38	140
	Susceptible Nomer pervi	Healthy	985±61	100
		Infected	1131±115	114
Cucumber—*Erysiphe cichoracearum*	Resistant Dalnevostochni	Healthy	2788±56	100
		Infected	3665±215	131
	Susceptible Nerosimi	Healthy	3025±114	100
		Infected	2645±65	86

Table 4

Comparison of the ability of ribosomes and high speed supernatant from healthy and infected tissues to incorporate C^{14}-leucine into proteins (counts/100 sec/1 mg RNA)

Ribosomes	Supernatant	Cabbage — *B. cinerea*		Cucumber — *E. cichoracearum*	
		Resistant	Susceptible	Resistant	Susceptible
Healthy	Healthy	1128	1031	2788	3052
Healthy	Infected	720	700	1017	1483
Infected	Infected	1713	1262	3665	2645
Infected	Healthy	1500	1138	1708	1698

gations. An increase of sensitivity to puromycine of the incorporation of C^{14}-leucine into protein by the ribosomes isolated from infected tissues also indicates the alterations in the properties of ribosomes (Table 5).

It has been postulated that alterations in the sensitivity of ribosomes to antibiotics is connected with conformational changes in their peptidile center (HULTIN, 1966, KULAEVA and KLACHKO, 1969). It is possible that analogous changes take place in the ribosomes under the influence of infection and this results in an increased sensitivity of the ribosomes to puromycine. The reaction of resistant

and susceptible varieties of cabbage to infection of the facultative parasite *Botrytis cinerea* differs quantitatively.

With the obligate parasite *Erysiphe cichoracearum* we demonstrated a qualitative difference in the reaction of resistant and sensitive varieties, respectively. As shown in Table 1, *E. cichoracearum* caused alterations in protein content. In the tissue of susceptible variety Nerosimi the protein content decreased while in the resistant variety Dalnevostochni it increased after infection.

Table 5

The effect of puromycine on the C^{14}-leucine incorporation by ribosomes in cellfree system (inhibition %)

Tissues	Cabbage – *B. cinerea*		Cucumber – *E. cichoracearum*	
	Resistant	Susceptible	Resistant	Susceptible
Healthy	22	35	64	20
Infected	36	50	80	40

These alterations in protein content reflect changes in the amount of ribosomes as well as in their functional activity. As is shown in Table 2, the amount of ribosomes increased in the tissues of the resistant variety and decreased in the susceptible one. The functional activity of these organelles was altered correspondingly (Table 3).

The data obtained indicate that upon infection the resistant varieties can synthesize proteins which may play a role in disease resistance (RUBIN and ARTSIHOVSKAYA, 1968, STAHMANN, 1965, LIPSITS, 1970). Susceptible varieties do not possess this ability.

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Defence Reaction Induced by Lysine in Rust-infected Wheat Leaves

By

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Wheat leaves fed with lysine by the leaf culture method and infected with *Puccinia recondita* exerted a necrotic type reaction (defence reaction) to the pathogen. This paper presents evidences for the hypothesis according to which altered amino acid metabolism of the host as a result of lysine administration is in connection with the experienced defence reaction to the fungus.

Previously it has been shown that rust-infected wheat leaves fed with lysine and other amino acids exert an inhibitory action on rust development (VAN ANDEL, 1966, DONCHEV, 1969). The aim of the present study was to determine the alterations in the amino acid content of wheat leaves fed by lysine during the period of the development of leaf rust *(Puccinia recondita)* infection, and to give an explanation for the defence reaction of wheat leaves to the leaf rust pathogen.

Materials and Methods

Leaves of the wheat cultivar Michigan Amber, healthy or infected with *Puccinia recondita*, race 21, were used in the present investigations. Leaf treatments with lysine and method of infection as well as incubation was described earlier (DONCHEV, 1969). The detached leaf culture method was applied according to WANG (1959). The amino acid contents were determined qualitatively by paper chromatography and quantitatively by a colorimetric method (HAIS and MACEK, 1963, BOJARKIN, 1956).

Results and Discussion

The results are presented in Fig. 1. From these investigations the following conclusions can be made:

1. The content of all of the amino acids investigated increases up to the second day after feeding with lysine. In the course of rust development (during the following days) some of the amino acids like arginine and pipecolic acid remain on an approximately constant level. The amount of other amino acids continues to

increase at a lower rate (glutamine and cysteine), or gradually decreases like lysine and glutamic acid.

2. It is noteworthy that during the period from the second to the fourth day the contents of lysine, glutamic acid and arginine quickly decrease.

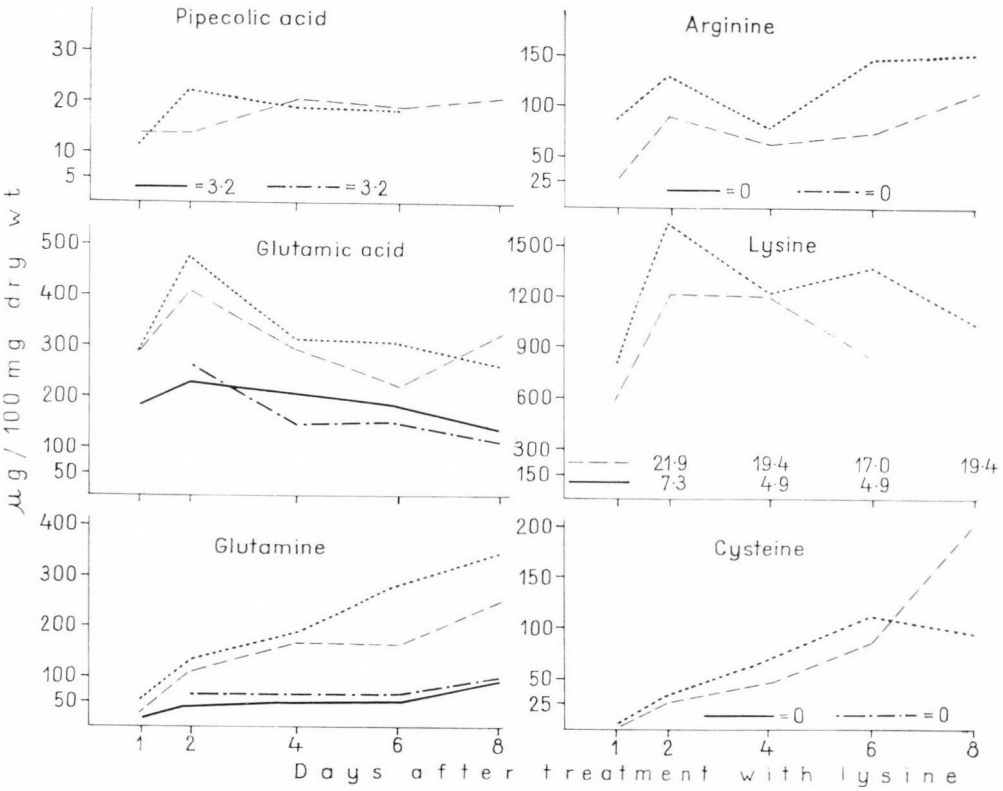

Fig. 1. Changes in the amounts of amino acids as a result of treatment of wheat leaves with lysine after leaf rust infection. ——— = healthy, non-treated; – – – – = healthy, treated with lysine; —·—·—· = rust-infected, non-treated; ..... = rust-infected, treated with lysine

3. Rust infection by itslef exerts a slight increase in the amount of lysine and glutamine, however has no effect on other amino acids. In fact, the level of glutamic acid in infected, non-fed leaves decreases below the level of this acid in the healthy, non-fed ones.

4. The curves of the healthy and rust-infected leaves fed by lysine have a similar slope, especially up to the fourth day.

5. The amounts of the amino acids in infected leaves fed by lysine exceed those in the healthy and fed ones.

6. The increase of the amount of pipecolic acid is perhaps the most interesting feature of the treatment with lysine. As is known, pipecolic acid is formed in plants as a result of the conversion of lysine (MAISTER, 1961). The significance of the accumulation of pipecolic acid in plant pathological reactions is treated in the book of GOODMAN, KIRÁLY and ZAITLIN (1967). As a result of the conversion of lysine in plant leaves α-keto-ε-aminocaproic acid, pipecolic acid and furthermore α-ketoglutaric acid are formed (KRETOWITSCH, 1961). The latter compound may play a role as an amino-group acceptor. Thereby, α-ketoglutaric acid has a primary importance in the transamination processes. The production of this keto-acid as an end-product of the conversion of lysine may give an explanation for the increase in the amounts of glutamic acid and glutamine.

Obviously, lysine fed to wheat leaves induces an alteration in the composition of amino acids in the host which is unfavourable for the pathogen. One can suppose that lysine as an inhibitor of the leaf rust fungus does not affect the pathogen directly, however, indirectly, by influencing amino acid metabolism in the host plant. Interestingly enough, the amounts of all of the amino acids in wheat leaves infected and fed with lysine exceed the amounts of the same amino acids in healthy leaves which were similarly treated with lysine. Certainly, the pathogen does not die after the adminstration of lysine in the host plant. There are no sings of any defence reaction in the host up to the sixth or seventh day after infection. Commencing the nineth day, however, a necrotic type reaction begins to develop around the infection sites (type O;) which is the result of the feeding of the leaves with lysine.

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Verlauf der Infektion bei den Weizenpflanzen, mit verschiedener Empfänglichkeit gegen *Erysiphe graminis* DC.

Von

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Results of microscopic studies on the process of infection of susceptible and resistant varieties of wheat (*Triticum aestivum* L.) inoculated by *Erysiphe graminis* f. sp. *tritici* Marchal, biotype 2 are summarized. Attention has been paid only to initial phases of infection comprising the period from inoculating the plant with conidia of the fungus till its fructification. Based on the morphogenesis of single organs of the fungus, the period studied in this paper has been divided into six stages: 1) conidial germination, 2) primary appressorium formation, 3) primary infection peg formation and penetration into epidermal cells, 4) haustorium formation and growth of the secondary air mycelium, 5) formation of the first fructification organs and 6) fructification. In the processes of the first three stages, no significant differences in the total dynamics of the fungus development were stated. A delay (suppression of growth) were seen only at the beginning of the fourth stage. In the following process in the resistant plants the suppression of the secondary air mycelium and haustorium formation were accompanied by the changes in the tissues near the epidermal cell. The experiments were carried out in air-conditioned boxes where temperature, relative air humidity and illumination were controlled.

Wachstum des Pilzes Erysiphe graminis an den verschieden anfälligen Wirtspflanzen verläuft ungleichmäßig. Bei den resistenten Pflanzen, im Vergleich mit den anfälligen Pflanzen, ist das Wachstum ausdrucksvoll gehemmt, eventuell ganz eingestellt. Aus dem Gesichtspunkt des Studiums mancher Grundfragen, die mit der Resistenz der Pflanzen zusammenhängen, ist es notwendig zu wissen, in welchem Zeitabschnitt der Pathogenese die angeführte Wachstumshemmung des Parasiten verläuft. Mit der Aufklärung dieser Frage befaßten sich bis jetzt mehrere Verfasser (CORNER, 1935, CHEREWICK, 1944, LUPTON, 1956, PAULECH, 1966, SMITH und BLAIR, 1950 und andere). In unserer Arbeit legen wir Ergebnisse des Studiums des Einflusses der verschieden anfälligen Wirtspflanzen des Weizens auf die Gesamtdynamik der Entwicklung des erwähnten Pilzes, im Verlauf der einzelnen Etappen des Infektionsprozesses vor.

Material und Methoden

Die Entwicklung des Pilzes *Erysiphe graminis* f. sp. *tritici* Marchal bewerteten wir an den Pflanzen von Winterweizen (*Triticum aestivum* L.), Sorte »Carsten V«, Befallstyp 4 (bewertet nach MAINS und DIETZ, 1930), an den Sommerwei-

zen, Sorte »Hope«, Befallstyp 3, »Weihenstephan Stam M_1«, Befallstyp 1, und an Haferpflanzen (*Avena sativa* L.), die keine Wirtspflanzen des betreffenden Pilzes sind. In der Entwicklungsstufe von zwei Blättern inokulierten wir die Versuchspflanzen mit den Konidien des Pilzes *E. graminis* f. sp. *tritici* Marchal (phy-

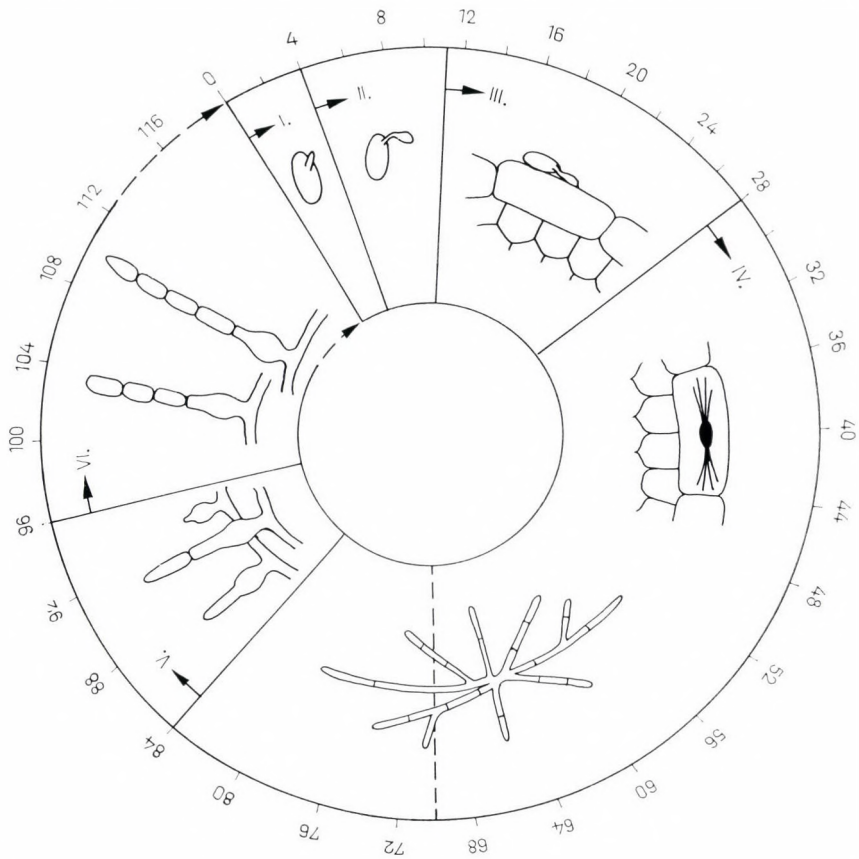

Abb. 1. Graphische Darstellung der einzelnen Etappen des Infektionszyklus. Die Ziffern längs des Umfangs des Kreises = die Stunden nach der Inokulation; O = Inokulation; I—VI = die Etappen des Infektionszyklus, die im Text beschrieben sind. Die Unterbrochene Linie = Ende der Inkubationszeit

siologische Rasse 2). In den gewählten Zeitabschnitten nahmen wir aus den ersten Blättern der inokulierten Pflanzen Proben zur Auswertung der Entwicklung des Parasiten.

Die Versuche wurden unter konstanten Bedingungen bei einer Temperatur von 20 °C ± 0.5 °C, einer relativen Luftfeuchtigkeit von 70% ± 5%, einer Beleuchtung $7 \cdot 10^{-2}$ cal cm^{-2} min^{-1}, und einer Lichttageslänge von 16 Stunden durchgeführt.

Ergebnisse

Auf Grund der Morphogenese der einzelnen Organe des Pilzes kann man den Zeitabschnitt von der Inokulation bis zur Fruktifikation des Pilzes in sechs Etappen wie folgt verteilen: 1) Konidienkeimung, 2) Ausbildung der primären Appressorien, 3) Bildung der primären Infektionsfäden und ihr Eindringen in die Epidermiszellen, 4) Haustorienbildung und Wachstum des sekundären Luftmyzels, 5) Bildung der ersten Fruktifikationsorgane, 6) Fruktifikation. Die angeführten Etappen repräsentieren im Grunde einen beendeten Infektionszyklus der Pflanzen mit den Konidien des betreffenden Pilzes. Im weiteren Verlauf der Pathogenese wird der Zyklus mehrfach wiederholt. Beginn und Zeitdauer der einzelnen Etappen werden in Abb. 1 dargestellt.

Tabelle 1

Verlauf der Keimung der Konidien des Pilzes *Erysiphe graminis* f. sp. *tritici* Marchal an den Blättern der verschieden anfälligen Wirtspflanzen ($\bar{x}$ ist Durchschnitt aus 5×100 ausgewerteten Konidien)

Stunden nach der Inokulation	Variante							
	Weizen						Hafer	
	Carsten V.		Hope		Weihenstephan		Český žltý	
	$\bar{x}$	$s_{\bar{x}}$	$\bar{x}$	$s_{\bar{x}}$	$\bar{x}$	$s_{\bar{x}}$	$\bar{x}$	$s_{\bar{x}}$
1	5.00	±1.75	4.60	±1.84	4.00	±1.00	10.00	±1.60
2	34.20	±2.20	39.30	±1.90	27.00	±2.10	26.20	±1.32
4	46.40	±2.50	51.70	±1.60	47.30	±1.50	48.15	±0.98
6	66.80	±2.20	65.00	±0.82	65.20	±2.20	67.00	±1.46
8	74.90	±1.30	76.60	±0.90	75.00	±1.60	73.80	±1.50
24	81.00	±1.06	78.00	±0.84	79.00	±1.17	78.00	±2.12

Der Beginn und die Zeitdauer der einzelnen Etappen des Infektionsprozesses waren an den verwendeten anfälligen und resistenten Pflanzen gleich. Nur die Intensität des Wachstums und Gesamtentwicklung des Parasiten wurden an den resistenten Pflanzen gehemmt. Von den Resultaten der Vergleichsstudien, verwirklicht im Verlauf der einzelnen Etappen, ist zu sehen, in welchen Etappen des Infektionsprozesses es zur Hemmung der Entwicklung des Parasiten und dadurch zur Verwendung der Resistenzelemente kam.

Die erste und verhältnismäßig kurze Etappe der Infektion beginnt mit der Inokulation der Pflanzen, sie setzt sich fort mit der Bildung der primären Appressorien. Ergebnisse des Studiums des Einflusses der anfälligen und resistenten Weizenpflanzen, sowie der Haferpflanzen, auf die Konidienkeimung, führen wir in Tabelle 1 und 2 an. Aus den angeführten Resultaten kann man sehen, daß die Keimung bei allen Varianten verläuft. Keine signifikanten Unterschiede im Pro-

Tabelle 2

Vergleich des Prozentsatzes der ausgekeimten Konidien des Pilzes *Erysiphe graminis* f. sp. *tritici* Marchal an den Blättern der verschieden anfälligen Weizen- und Haferpflanzen
(Ausgewertet nach 24 Stunden nach der Inokulation)

Verglichene Varianten	$\bar{x}-\bar{x}$	$\bar{d}$	t(8)	P
Carsten V.—Hope	81—78	3.00	2.22	—
Carsten V.—Weihenstephan	81—79	2.00	1.33	—
Carsten V.—Český žltý	81—78	3.00	1.27	—
Weihenstephan—Český žltý	79—78	1.00	0.41	—
Weihenstephan—Hope	79—78	1.00	0.71	—
Český žltý—Hope	78—78	0.00	0.00	—

— = $P < 0.05$

Tabelle 3

Verlauf der Appressorienbildung des Pilzes *Erysiphe graminis* f. sp. *tritici* Marchal an den Blättern der verschieden anfälligen Pflanzen
($\bar{x}$ ist Durschnitt aus 5×100 ausgewerteten Konidien)

Stunden nach der Inokulation	Variante							
	Weizen						Hafer	
	Carsten V.		Hope		Weihenstephan		Český žltý	
	$\bar{x}$	$s_{\bar{x}}$	$\bar{x}$	$s_{\bar{x}}$	$\bar{x}$	$s_{\bar{x}}$	$\bar{x}$	$s_{\bar{x}}$
4	3.8	±1.60	4.5	±1.20	5.2	±1.42	4.0	±0.98
6	36.0	±1.45	40.0	±1.93	42.0	±1.04	38.2	±2.10
8	61.7	±1.03	60.2	±1.90	58.8	±1.96	57.6	±1.35
10	70.3	±1.50	71.0	±1.40	69.0	±0.78	68.4	±0.72
12	76.0	±1.59	75.0	±1.60	77.2	±0.69	76.0	±1.65
24	79.0	±1.41	77.0	±1.40	78.0	±0.98	78.0	±1.20

zent der ausgekeimten Konidien an den anfälligen und resistenten weder Weizenpflanzen, noch Haferpflanzen wurden gefunden. Die Gesamtdynamik der Konidienkeimung war bei den verwendeten Varianten gleich. In dieser Etappe des Infektionsprozesses kam es zu keiner Äußerung von Resistenzelementen.

Bei optimalen und ihnen nahen Bedingungen beginnt nach vier Stunden nach der Inokulation an den Pflanzen die zweite Etappe des Infektionsprozesses zu verlaufen. Diese Etappe wird als Bildung der primären Appressorien bezeichnet. Die Resultate der vergleichenden Studien dieser Etappe führen wir auf den Tabellen 3 und 4 an. Aus den Ergebnissen können wir feststellen, daß die gesamte Dynamik der Appressorienbildung an den anfälligen, sowie auch an den resistenten Weizen- und Haferpflanzen gleich verlief. In dieser Zeit, ähnlich wie in der

Tabelle 4

Vergleich des Prozentsatzes der ausgebildeten Appressorien bei dem Pilz *Erysiphe graminis* an den Blättern der verschieden anfälligen Weizen- und Haferpflanzen (Ausgewertet nach 24 Stunden nach der Inokulation)

Verglichene Varianten	$\bar{x}-\bar{x}$	$\bar{d}$	t(8)	P
Carsten V.—Hope	79—77	2.0	1.05	—
Carsten V.—Weihenstephen	79—78	1.0	0.58	—
Carsten V.—Český žltý	79—78	1.0	0.58	—
Weihenstephan—Český žltý	78—78	0.0	0.00	—
Weihenstephan—Hope	78—77	1.0	0.58	—
Český žltý—Hope	78—77	1.0	0.55	—

— = $P < 0.05$

Etappe der Konidienkeimung, kamen bei den resistenten Pflanzen die Elemente der Resistenz nicht ausdrucksvoller zur Geltung. In der Anzahl der gebildeten Appressorien bei den einzelnen Varianten wurden keine signifikanten Unterschiede gefunden. Alle ausgekeimten Konidien bildeten Appressorien aus. Bei den Konidien, die mit zwei oder mehr Keimungsfäden keimten, kam es zur Bildung der typischen Appressorien normalerweise nur bei einem Faden. Die Beendigung der Bildung der primären Appressorien wurde nach 8, eventuell nach 10 Stunden nach der Inokulation beobachtet. Als entwickelte erwachsene Appressorien erachteten wir die, bei denen zwischen dem Keimungsfaden und dem eigentlichen Appressorium eine Sperre ausgebildet wurde. Im Termin des Beginns und der Beendigung der Bildung der Appressorien bemerkten wir an den verschieden anfälligen Pflanzen keine signifikanten Unterschiede. Abb. 2 zeigt die gesamte Dynamik der Konidienkeimung und der Bildung der primären Appressorien, sowie auch ihre zeitliche Kontinuität.

Nach der Gestaltung der Appressorien bildet der Pilz an den inokulierten Infektionsfäden, die durch die Kutikula und Wände der epidermalen Zellen in die Protoplasten dringen, aus. An den Blättern des Hafers wurde die Entwicklung des Pilzes im Stadium der Appressorien eingestellt. In manchen Fällen wurde auch Penetration verwirklicht, aber der Parasit setzte sie schon in weiterer Entwicklung nicht fort. Die Mechanismen, durch die es zur Einstellung seiner Entwicklung kam, sind nicht bekannt. In welchem Ausmaß die physikalischen oder chemischen Faktoren dabei zur Geltung kamen, kann man nicht eindeutig sagen. Am wahrscheinlichsten bei der Einstellung der Entwicklung des Parasiten in dieser Etappe des Infektionszyklus, wirken beide Gruppen der Faktoren. An den Weizenblättern kam es bei allen Varianten zur Ausbildung der Infektionsfäden und deren Durchdringen in die epidermalen Zellen. Die Bildung der ersten Infektionsfäden wurde annähernd nach 10—12 Stunden, und die Beendigung der Penetration etwa nach 14 Stunden nach der Inokulation beobachtet.

Nach dem Durchdringen der Infektionsfäden in die epidermalen Zellen, begann der Parasit Haustorien, und an der Oberfläche der Blätter das sekundäre Luftmyzel auszubilden. An den Blättern der Sorte »Weihestephan« kam es zur Ausbildung der fingerförmigen Haustorien nicht. Im Gebiet des Durchdringens des Parasiten wurden im Protoplasma kleine Grana beobachtet. Die ganze Zelle wurde nach dem Durchdringen des Parasiten dunkel gefärbt. Die Entwicklung des Parasiten wurde in diesem Stadium des Infektionsprozesses wegen der Hypersensitivität der befallenen epidermalen Zellen eingestellt.

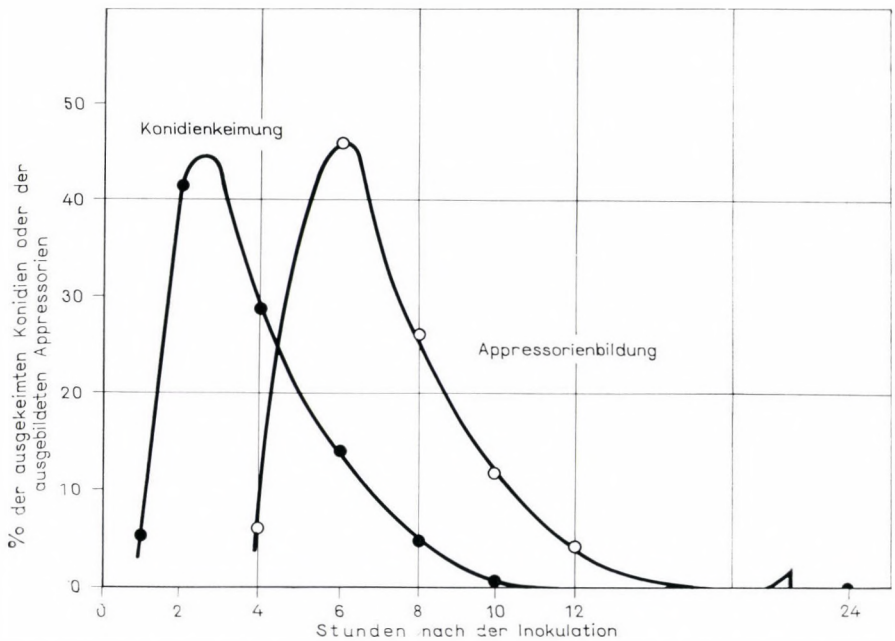

Abb. 2. Dynamik der Konidienkeimung und Bildung der primären Appressorien und ihre Zeitfolge

An den Weizenblättern der Sorte »Carsten V« und »Hope« wurden die Infektionsfäden verbreitet, sie bildeten ein kugelförmiges und birnenförmiges Gebilde aus. Dieses Gebilde wurde fortschreitend größer, änderte seine Form, orientierte sich mit Längsachse in der Richtung der Länge des Blattes, bis aus ihm ein Körper des Haustoriums entstand. An beiden Enden des Körpers des Haustoriums begannen fingerförmige Ausläufer anzuwachsen. Der Körper des Haustoriums wurde etwa nach 18–20 Stunden, und die erwachsenen Haustorien nach 33–36 Stunden nach der Inokulation ausgebildet.

Die ersten Zellen des sekundären Luftmyzels bildeten sich annähernd nach 18–20 Stunden nach der Inokulation aus. Nach diesem Zeitabschnitt werteten wir bei beiden Sorten die Anzahl der Haustorien und der Zellen des sekundären

Luftmyzels in den Infektionshöfen, nach den sechsstündigen Zeitabschnitten, aus. Präparate für die mikroskopische Beobachtung wurden in Laktophenol mit Anilinblau vorbereitet (WHITE und BAKER, 1954). Die Ergebnisse dieser Arbeiten werden in Abbildung 3 und 4 dargestellt. Aus den angeführten Diagrammen ist zu ersehen, daß an der stark anfälligen Weizensorte »Carsten V« die

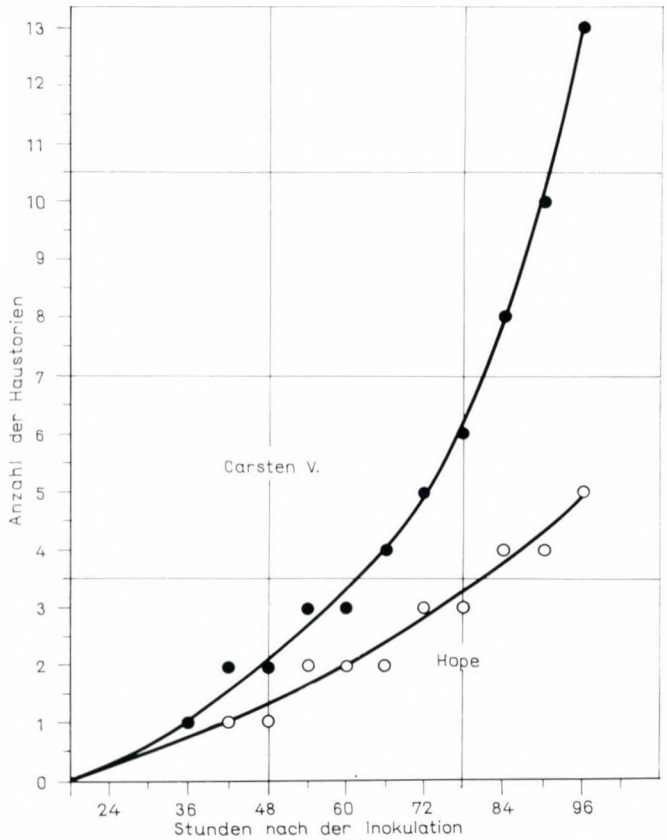

Abb. 3. Dynamik der Haustorienbildung an den anfälligen und resistenten Weizenpflanzen (durchschnittliche Werte aus 20—40 Infektionshöfen)

Entwicklung des Parasiten intensiv verläuft. Die durchschnittliche Anzahl der Haustorien des Parasiten, sowie auch die Anzahl der Zellen des sekundären Luftmyzels in den Infektionshöfen, wird schnell erhöht. Bei der Sorte »Hope« wird die Entwicklung des Parasiten ausdrucksvoll gehemmt. Aus den Resultaten, die beim Studium dieser Etappe des Infektionsprozesses gewonnen wurden, kann man folgern, daß es zu ausdrucksvoller Anwendung der Elemente der Resistenz erst nach der definitiven Anknüpfung der physiologischen Verbindung des Parasiten mit der Wirtspflanze kam. Es handelt sich hier um Resistenz der metabolischen

Natur. Die Hemmung der Entwicklung des Parasiten beginnt erst während der Periode, wenn zwischen den Haustorien und dem Protoplasma ein intensiver Austausch der Stoffe verläuft. Dieser Typ der Rezistenz, der durch den Parasiten induziert wird, äußert sich erst nach der Bildung eines, eventuell mehrerer Haustorien im Infektionshof.

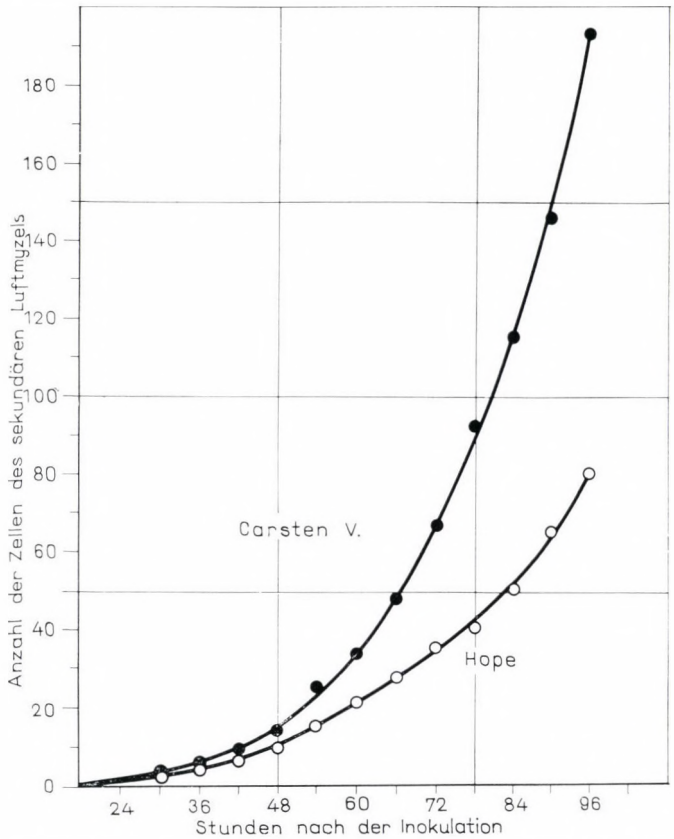

Abb. 4. Dynamik des Wachstums des sekundären Luftmyzels an den anfälligen und resistenten Weizenpflanzen (durchschnittliche Werte aus 20–40 Infektionshöfen)

Im Verlauf der weiteren Entwicklung des Pilzes beobachteten wir Bildung der ersten Fruktifikationsorgane, als der fünften Etappe des Zyklus der primären Infektion. Die ersten Basal- und Mutterzellen der Konidienträger begannen sich an dem Luftmyzel nach 82–86 Stunden nach der Inokulation zu bilden. Das war etwa 12 Stunden nach der Inkubationszeit der Beendigung. Bei der Sorte »Carsten« war die Anzahl der Konidienträger, die an dem Luftmyzel entstanden, höher als an der Sorte »Hope'«.

An den Konidienträgern kam es zur Bildung der Konidien, die nach dem

Ausreifen und nach dem Freiwerden aus den Konidienträgern neue Infektionen auszurufen imstande waren. Bei den Bedingungen unserer Experimente beobachteten wir den Beginn der letzten Etappe des Zyklus der primären Infektion annähernd 96 Stunden nach der Inokulation. Im Verlauf der weiteren Entwicklung, fruktifizierte der Pilz an der Sorte »Carsten V« intensiv, und an der Sorte »Hope« wurde die Fruktifikation ausdrucksvoll gehemmt.

Zum Schluß können wir behaupten, daß die Entwicklung des Pilzes *Erysiphe graminis* an den resistenten Pflanzen in verschiedenen Etappen des Infektionszyklus, gehemmt, oder ganz eingestellt werden kann. Die Mechanismen, mittels derer die Pflanzen die Entwicklung des Parasiten hemmen, sind bei den verwendeten Pflanzen nicht gleich. Das Studium der Resistenz des Weizens gegen den Pilz *E. graminis* muß man darum an mehreren Sorten, eventuell Arten mit verschiedenem Anfälligkeitsgrad durchführen.

Zusammenfassung

In dieser Arbeit werden Ergebnisse der mikroskopischen Studien des Infektionsprozesses der anfälligen und resistenten Weizensorten (*Triticum aestivum* L.), die mit *Erysiphe graminis* f. sp. *tritici* Marchal (physiologische Rasse 2) inokuliert wurden, zusammengefaßt. Auf Grund der Morphogenese der einzelnen Organe des Pilzes wurde der Zeitabschnitt von der Inokulation bis Fruktifikation wie folgt verteilt: 1) Konidienkeimung, 2) Ausbildung der primären Appressorien, 3) Bildung der primären Infektionsfäden und ihr Eindringen in die Epidermiszellen, 4) Haustorienbildung und Wachstum des sekundären Luftmyzels, 5) Bildung der ersten Fruktifikationsorgane, 6) Fruktifikation. Bei den einzelnen Etappen wurden der Beginn, die Zeitdauer und Gesamtdynamik festgestellt. Bei den Pflanzen mit verschiedenem Resistenzgrad wurden die Etappen, während derer es zur ausdrucksvollen Hemmung, eventuell gänzlichen Einstellung der Entwicklung des Parasiten kam, limitiert.

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A Biochemical Basis for the Defence Reaction of Tomato to *Cladosporium fulvum*

By

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A hypothesis is outlined according to which the hypersensitive reaction (resistance) is brought about by specific excretion products of the fungus destroying the cell structure in the resistant plant but not in the susceptible one. Experiments in favour of this hypothesis is presented as far as resistance by the genes Cf 1 and Cf 2 is concerned. Discs were prepared from tomato leaves which had taken up $^{32}PO_4$ through the petiole. These discs were infiltrated with a buffered high molecular weight fraction of the culture filtrate of *C. fulvum*. The leakage of the labelled compound from the cells was measured to assess toxicity of the fungal products. Products from a fungal race which was able to attack the test plant (susceptible host-parasite combination) did not influence the leakage. In contrast, excretion products from a fungal race unable to attack the test plant (resistant host-parasite combination) caused accelerated leakage of the tomato cells. Thus a correlation was found between the occurrence of the hypersensitive reaction *in vivo* and the acceleration of the leakage of tomato cells in model experiments. It is suggested that leakage is related to the destruction of the cell membranes following contact with fungal excretion products. The role of Cf genes in the host as well as of the avirulence genes in the fungus is discussed in this mechanism.

Most tomato varieties are susceptible to *Cladosporium fulvum*. This fungus attacks the leaves of tomato, especially at the lower side. In the case of resistance the plant reacts to inoculation with a hypersensitive reaction.

Genetically, resistance of tomato to *Cladosporium* is determined by four or five independent genes (Cf 1, Cf 2, etc.) in such a way that each of these genes confers only resistance to certain of the races of the pathogen (DAY, 1954, HUBBELING, 1966). This complicated situation is illustrated in part in Table 1.

Tomato LMR with the dominant resistance gene Cf 1 is resistant to attack by race 0 and 2, but susceptible to the races 1 and 1.2. On the other hand, the tomato variety Vetomold with the dominant resistance gene Cf 2 is resistant to race 0 and 1, but not to 2 and 1.2. The tomato Moneymaker which is recessive to all resistance genes is susceptible to all races of the pathogen. This scheme can be extended with the resistance genes Cf 3 and Cf 4, possibly Cf 5 (HUBBELING, 1970).

The pattern of resistance and suceptibility at first sight looks very complicated. You may wonder why we tried to unravel the biochemical mechanism of resistance to this disease. The reason was the following:

A very similar relation between host varieties and fungal races exists in certain other plant diseases like apple scab caused by *Venturia inaequalis*, potato

late blight caused by *Phytophthora infestans*, flax rust and other rust diseases. A former guest-worker of our institute (Dr. RAA) has studied the biochemical mechanism of one of these diseases, namely of apple to *Venturia* (RAA, 1968a, b,

Table 1

Relation between some tomato varieties and races of *Cladosporium fulvum*

Tomato variety	Dominant resistance genes	*Cladosporium fulvum*, physiologic race			
		0	1	2	1.2 → etc.
Moneymaker	None	S	S	S	S
LMR	Cf 1	R	S	R	S
Vetomold	Cf 2	R	R	S	S
V 473	Cf 1 Cf 2	R	R	R	S
V 121	Cf 3	R	R	R	R
59 R	Cf 1 Cf 3	R	R	R	R
↓					
etc.					

R = resistant
S = susceptible

Table 2

Presumed toxin production by *Cladosporium fulvum*

Tomato variety	Dominant resistance genes	*Cladosporium fulvum*, physiologic race			
		0	1	2	1.2
		Presumed genotype for toxin production			
		A_1A_2	a_1A_2	A_1a_2	a_1a_2
Moneymaker	None	S	S	S	S
LMR	Cf 1	R	S	R	S
Vetomold	Cf 2	R	R	S	S
V 473	Cf 1 Cf 2	R	R	R	S

gene A_1 = ability to form a toxin for tomato Cf 1
gene A_2 = ability to form a toxin for tomato Cf 2

RAA and KAARS SIJPESTEIJN, 1968). He has provided strong evidence that *Venturia* excretes a compound into the medium which is toxic selectively to resistant apple varieties in such a way as to cause cell damage and a hypersensitive reaction. In contrast this compound is not toxic to apple varieties which are susceptible to this same strain of *Venturia*

We now reasoned that the hypersensitive resistance reaction in tomato

might be effected in a similar way as in apple (VAN DIJKMAN and SIJPESTEIJN). Our hypothesis suggests that race 1 has the ability to produce a compound which is toxic selectively to a tomato variety which it cannot attack like Vetomold or V 473 with the gene Cf2, but if it should not be toxic to a susceptible variety like Moneymaker or LMR which lack the gene Cf2. The pattern of resistance towards race 2 is the reverse. Therefore we suppose that this race produces another compound which is toxic selectively to tomato LMR or V 473 with gene Cf1 giving a hypersensitive reaction; but it should not be toxic to the susceptible varieties Moneymaker or Vetomold which lack Cf 1.

In Table 2 we have indicated with A_1 the presumed ability to form a compound toxic to tomato Cf 1 and with A_2 the ability to form a compound toxic to tomato Cf 2. In the scheme these abilities have tentatively been written as a genotype. Race 0 cannot attack tomatoes which possess either gene Cf 1 or Cf2; therefore it might produce both toxins.

To obtain evidence for our hypothesis of the production of various selective toxins by the fungus, model experiments were carried out in which the toxicity of culture fitrates of the various races towards different tomato varieties was studied (VAN DIJKMAN and KAARS SIJPESTEIJN). In the first experiment the races a_1A_2 and A_1a_2 and the tomatoes LMR and Vetomold were studied.

Methods

The experiment was carried out in the following way. In order to obtain the compounds excreted by the fungus, shake cultures of the two races were grown for about 3 weeks. Thereafter the culture filtrate was freed from low molecular material by filtration over Sephadex G-25. The high molecular weight fraction was freeze-dried and stored until use. Meanwhile tomato plants were grown of the varieties LMR and Vetomold. Mature leaves were cut off and placed for several hours with their petioles into a solution of $^{32}PO_4$ to obtain a labelling of the leaf cells. Then discs were prepared from these leaves. They were transferred to a buffered solution of the fungal material. To ensure proper contact between the labelled plant cells and the fungal material the liquid was infiltrated into the dices by evacuation of the flask. After infiltration air was slowly admitted again.

We expected that the infiltrated fungal material would contain a compound toxic selectively to the cells of a resistant host plant but not to those of a susceptible host plant. Hence we expected that in the case of resistance the host cells would be damaged and that consequently the labelled compounds would leak out of the cells and out of the discs into the surrounding liquid. But in the case of susceptibility we expected no leakage by fungal products. And this is indeed what we found. The amount of leakage was determined by measuring radioactivity of the liquid at different intervals after infiltration. The percentage of leakage of the total ^{32}P present could be calculated.

Results and Discussion

Figures 1A and 1B give the results obtained with tomato LMR (Cf1) and with Vetomold (Cf2).

The extent of leakage of labelled compounds from the discs is given in relation to the time elapsed after infiltration of the excretion products of the fungi.

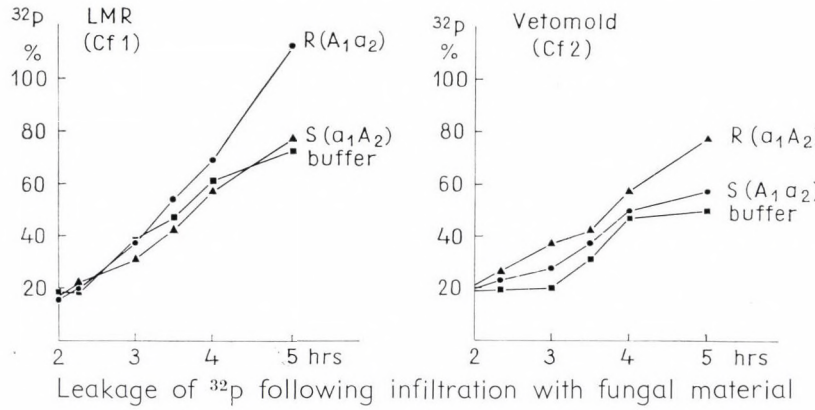

Fig. 1. A and B

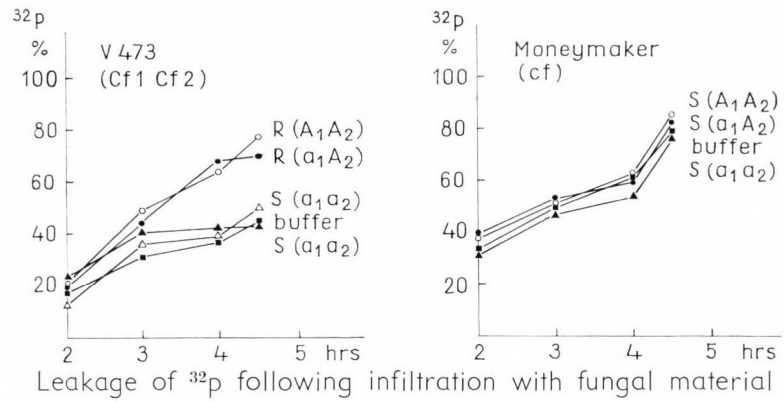

Fig. 2. A and B

LMR (Cf 1) is susceptible to the race a_1A_2 and infiltration with products of this race does not cause a leakage which exceeds that caused by infiltration of the buffer solution alone. The same fungal products, however, cause strong leakage in excess of the buffer control after infiltration into Vetomold (Cf 2), which is resistant to this race a_1A_2. Quite the opposite situation was found for the race A_1a_2 which is

able to attack Vetomold (Cf 2) but not LMR (Cf 1) : we found strong leakage of LMR but not of Vetomold. These results are strongly in favour of our hypothesis of selectively toxic products being excreted by the parasite.

A similar correlation between resistance *in vivo* and leakage in the model experiment was found for the tomato variety V 473 which carries the resistance genes Cf 1 and Cf 2. (Fig. 2A) Again infiltration with material from two fungal

Table 3

Observed correlation between resistance in vivo and leakage by selective toxins

Tomato variety	Dominant resistance genes	*Cladosporium fulvum*, physiologic race			
		0	1	2	1.2
		Proposed genotype for avirulence			
		A_1A_2	a_1A_2	A_1a_2	a_1a_2
Moneymaker	None	S/N	S/N	S	S/N
LMR	Cf 1	R	S/N	R/L	S
Vetomold	Cf 2	R	R/L	S/N	S
V 473	Cf 1 Cf 2	R/L	R/L	R	S/N

R = resistant
S = susceptible
L = leakage
N = no leakage

races to which this plant is resistant – in this case A_1A_2 and a_1A_2 – causes strong leakage in excess of the control. In contrast the high molecular weight material excreted by two isolates (a_1a_2) which are able to attack this tomato variety do not cause any extra leakage.

From three of these races the products were also infiltrated into the tomato Moneymaker which is suceptible to all races of the pathogen; it is recessive to all resistance genes. According to expectation no leakage in excess of the control was observed (Fig. 2B).

Table 3 summarizes our results. It gives the host-parasite relation for a number of varieties and races as well as the results of the leakage experiments in the various combinations studied. It is apparent from these results that there is a correlation between the occurrence of a hypersensitive resistance reaction *in vivo* (R) and leakage in the model experiments (L) and also between susceptibility (S) and the absence of leakage (N). This correlation suggests strongly that the various physiologic races of *Cladosporium* are distinguished by their ability to produce one or more compounds which each are toxic selectively only to those tomato varieties which carry the appropriate resistance gene.

Possibility is limited to go at length into the genetic implications of these findings. I would only like to indicate that we believe that the various resistance genes in the tomato control the presence of various receptor sites in the cell mem-

branes; each of these sites should be sensitive selectively to one of the fungal toxins only. The presumed genes in the fungus which control toxin production may be called avirulence genes. Each of these corresponds to a resistance gene in the tomato host. Up till now we have only studied the action of *Cladosporium* fractions on tomatoes with the genes Cf 1 and Cf 2.

As I mentioned in the beginning the tomato has 4 to 5 different resistance genes. To these may correspond 4 to 5 avirulence genes in the parasite. Our future work will also involve tomatoes with the other resistance genes.

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Cyclamin, an Antifungal Resistance Factor in *Cyclamen* Species*

By

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Cyclamen persicum contains the saponin cyclamin, which has an antifungal property. The role of this saponin in disease resistance was studied. There is no degradation of cyclamin in the homogenized plant material, even after 24 hr incubation at room temperature. This indicates that the *Cyclamen* species do not have saponin-degrading enzymes. Artificial inoculation with *Botrytis cinerea* revealed that the fungus can only develop on the stems but not on the leaves. This difference can be explained by the saponin contents of the respective organs. Only those fungi which can produce a saponin-degrading enzyme can be expected to settle successfully on this plant species. Results are presented on the basis of which cyclamin has to be regarded as a potent factor in the defence mechanism of *Cyclamen* species against fungal invasion.

Saponins occur as normal constituents in more than 70 plant families (PARIS, 1963), in concentrations ranging from 0.1 to 10% of the dry weight (KOFLER, 1927). Since these compounds have also antifungal properties, 5–200 μg/ml were sufficient to kill all fungal species tested (TSCHESCHE and WULFF, 1964, WOLTERS, 1968), it was interesting to study whether saponins play any role in the defense mechanism of plants against fungal invasion.

The studies reported by TURNER (1953, 1956, 1960) and ARNESON and DURBIN (1967, 1968) are an indication that saponins must be considered as factors in host–parasite interactions. But before general conclusions can be drawn regarding the importance of saponins as a group, more host–parasite combinations need to be studied. The following is a preliminary report on the role of cyclamin in *Cyclamen persicum* in regard to fungal infections.

Materials and Methods

Cyclamin: Pure cyclamin, prepared from *Cyclamen europaeum*, was kindly supplied by Dr. G. WULFF (Institute of Organic Chemistry and Biochemistry, University of Bonn).

The fungal species used in this study were from the following sources. Centraalbureau voor Schimmelcultures, Baarn: *Cryptocline cyclaminis* (202.59),

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Fusarium oxysporum f. cyclaminis (159.57), *Ramularia cyclaminicola* (399.51), *Septoria cyclaminis* (120.43). *Botrytis cinerea* was isolated from infected *Cyclamen* plants. All other fungi were from the culture collections of either the Institut für Pflanzenkrankheiten, Bonn or the Plant Pathology Section, Beirut.

Hemolysis test. The packed red blood cells were prepared from citrated bovine blood as described earlier (SCHLÖSSER and GOTTLIEB, 1966a). The test suspension consisted of one ml packed red blood cells in one litre of isotonic (0.9%) sodium chloride solution. Sixty minutes after the addition of the material to be tested it was visually determined whether hemolysis had occurred.

Quantitative cyclamin determination. Plant juice was obtained from the different tissues by means of a hand press. Using the dilution technique it was determined at which dilution hemolysis still occurred. The amount of cyclamin present in the original juice was calculated from the degree of dilution and the known detection limit of cyclamin (3 μg/ml).

Surface deposit and diffusion. In order to test whether there is a deposit of cyclamin on the leaf surface or whether diffusion of this saponin can occur, filter paper discs (5 mm diameter) were placed on the surface of intact leaves. These discs were kept moist for 5 hours after which they were used directly in the hemolysis test. Similarly, such discs were also placed on wounded (scratched) surfaces of leaves and were tested under the same conditions for absorption of cyclamin.

Sensitivity of fungi. To know the sensitivity of different fungal species cyclamin was incorporated into glucose-yeast extract-agar (10g/2g/15g/l) at intervals of 10 μg/ml. After incubation at 20°C for different lengths of time it was checked at which cyclamin concentration the mycelial growth was completely inhibited (LD_{100}).

Infection experiments. In November 1967 *C. persicum* plants of the cultivar "Ruhm von Wandsbeck" were inoculated and incubated under green house conditions. On stems a slight cut was made into which a small piece of mycelium from *Botrytis cinerea* was placed. After one week the percentage of positive infections was determined. The plant material from the infection experiment was also used for the isolation of *B. cinerea* on glucose-yeast extract-agar plates. On leaves parts of the surface were scratched off onto which spots small pieces of mycelium of the same fungus were placed. One week later the attempt was made to isolate *B. cinerea* from the points of inoculation.

Cyclamin inactivation. One part of corm tissue of *C. persicum* (wild type) was homogenized in a Waring Blendor with the addition of nine parts of isotonic sodium chloride solution. The liquid was decanted, autoclaved without any loss of activity and kept as a stock solution. For experiments, one part of this stock solution was diluted with two parts glucose-yeast extract-medium. Before and after autoclaving this medium contained 1.500 μg of cyclamin/ml, confirming again the stability of this saponin. To each test tube, containing two ml of this cyclamin medium, a disc of fungal mycelium (5 mm diameter) was added which had been

cut from agar plates. After four days of incubation on a reciprocal shaker (80 strokes/minute) the fungal growth was checked and the cyclamin in the medium was determined by means of the hemolysis test.

Enzymatic degradation of cyclamin by Botrytis cinerea. A *Cyclamen* specific strain of *B. cinerea* was cultured on a liquid glucose-yeast extract medium which contained 1.500 μg cyclamin/ml, originating from the corm stock solution. After

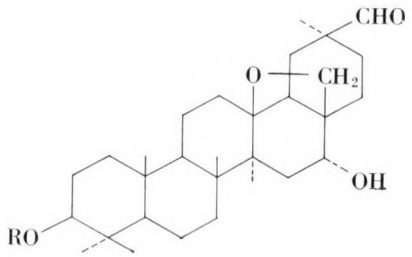

Fig. 1. Structure of cyclamin (after TSCHESCHE et al., 1969)
R = Arabinose—Glucose—Glucose
|
Glucose Xylose

four days of incubation on a rotary shaker the culture filtrate and the mycelium were separated. The wet mycelium (5.5 g) was ground with sand (5.5 g) in a mortar with the addition of 11 ml distilled water. The fine paste obtained was centrifuged for 15 minutes at 7.000 r.p.m.. The resulting particulate fraction (pellet) was once washed with 11 ml of isotonic sodium chloride solution (0.9 %) and then suspended in 11 ml of the same medium. To two ml of corm stock solution 0.5 ml particulate fraction (made up to one ml with water) or one ml of each the culture filtrate or the supernatant fraction were added. In each case the mixture contained 1.500 μg of cyclamin/ml. After 1,2 and 15 hours of incubation on a rotary shaker the cyclamin retained in the medium was determined.

Results and Discussion

Saponins in Cyclamen species

Most of the chemical work was done on the saponins of *Cyclamen europaeum*. TSCHESCHE et al. (1969) found a number of chemically closely related compounds present in the different plant parts of this *Cyclamen* species, but one of these accounted for about 90 % of the total saponin content. This compound, commonly called cyclamin (Fig. 1), is also found in *C. persicum*. As the different cyclamin derivatives in this *Cyclamen* species have not been identified we used pure cyclamin as a reference, therefore, all values given are cyclamin equivalents. This generalization is justified because the natural cyclamin derivatives differ very little in their physiological activity (SCHLÖSSER and WULFF, 1969).

Cyclamin distribution in plant parts

Using the hemolysis test we determined the cyclamin content in different plant parts of both the wild *C. persicum*, which is widely distributed in the mountain region of Lebanon and a cultivated form of *C. persicum* (Ruhm von Wandsbeck). Roots, corms and leaves of both forms contained considerable amounts of cyclamin (Table 1), ranging between 1.200 and 100.000 μg per milliliter of plant juice.

Table 1

Cyclamin content of different plant parts of both, the cultivated ("Ruhm von Wandsbeck") and the wild type of *Cyclamen persicum*

Plant part	μg cyclamin per ml plant juice	
	cultivated type	wild type
Root	5000–8300	–
Corm	1250–2500[a]	50.000–100.000[b]
Stem	50–90	20
Leaf	5000–6000	6300

[a] Hazelnut-sized corm, seven month old
[b] Corm 10–15 cm in diameter, several years old

The differences between the cultivated and the wild type were not significant. In the case of the corms the physiological age is responsible for the difference in cyclamin content. The corms of the cultivated *C. persicum* were 6–8 months old and had the size of a hazel nut, whereas the corms from the wild form were several years old and were 10–15 cm in diameter. The stems of both forms were virtually free of saponin.

Cyclamin was detectable only after the cells had been broken, there was no saponin deposit on the intact leaf surface and no diffusion occurred from intact cells. This finding is not unexpected. Saponins react with all cell membranes which contain sterols (SCHLÖSSER, unpublished). This means that the saponins would destroy the membranes of the cells in which they are confined if they would float freely in the cytoplasm. Therefore in intact cells these compounds have to be stored in compartments, most probably the vacuoles, from which they are released when the cells are damaged.

When incubated for different time intervals the cyclamin concentration in plant homogenates remained unchanged (Table 2). This indicated that all the saponin was immediately available in its active form and that no activation process was involved.

Even under non sterile conditions at room temperature the cyclamin concentration in plant homogenates remained stable within 22 hours, which can only be explained by the lack of saponin degrading enzymes in the host tissue.

Table 2

Stability of cyclamin in the plant juice after homogenization

Material	Incubation time at room temperature in hours	μg cyclamin per ml plant juice
C. persicum (cultivated type)		
Root	0[a]	8300
	1	8300
	2	8300
Corm	0[a]	1250
	1	1250
	2	1250
Stem	0[a]	80
	1	80
	2	80
Leaf	0[a]	5000
	1	5000
	2	5000
C. persicum (wild type)		
Leaf	0[a]	6250
	22	6250

[a] The plant juice was boiled within one minute after extraction.

Sensitivity of fungi to cyclamin

When the LD_{100} of several fungal species was determined (Table 3) it was found in all cases that less than 200 μg cyclamin/ml were sufficient to inhibit myce-

Table 3

Cyclamin concentration required for the complete inhibition of growth of several fungi

Species	μg/ml	Species	μg/ml
Ophiobolus graminis	30	*Aphanomyces laevis*	90
Sclerotinia cinerea	30	*Cylindrocarpon radicicola*	110
Botrytis tulipae	50	*Trichoderma viride*	110
Didymella lycopersici	50	*Phycomyces blakesleanus*	110
Trichothecium roseum	50	*Fusarium nivale*	130
Colletotrichum hedericola	50	*Aspergillus niger*	150
Phoma betae	90	*Alternaria tenuis*	170
		Fusarium oxysporum	170

lial growth completely. The magnitude was about the same as that reported by TSCHESCHE and WULFF (1964) and WOLTERS (1968).

Only species belonging to the *Pythiaceae*, like *Pythium* and *Phytophthora*, were resistant to cyclamin (Table 4). These fungi lack sterols in their membrane system (SCHLÖSSER and GOTTLIEB, 1966b, 1968, SCHLÖSSER et al., 1969) which

Table 4

Resistance of *Pythium* and *Phytophthora* species to cyclamin

Fungal species	LD_{100} (μg/ml)
Ophiobolus graminis	30
Colletotrichum hedericola	50
Pythium ultimum	>200[a]
P. irregulare	>200[a]
P. debaryanum	>200[a]
Phytophthora cactorum	>200[a]
P. citrophthora	>200[a]
P. cinnamoni	>200[a]

[a] at 200 μg cyclamin/ml the mycelial growth was unaffected and like that of the untreated controls.

are required for the membranolytic action of saponins (SCHLÖSSER, 1971), hence their resistance to these compounds. In many respects saponins resemble the polyene antibiotics which also require the presence of sterols in cell membranes for their membranolytic effect (SCHLÖSSER and GOTTLIEB, 1966a, b).

Cyclamin inactivation by fungi

Despite the high cyclamin concentration in corms and leaves of about 5.000 to 50.000 μg/ml plant juice, which is approximately 25 to 1000 times the LD_{100} for all fungi tested, some fungi, such as *Cryptocline cyclaminis*, *Fusarium oxysporum* f. *cyclaminis*, *Glomerella cingulata*, *Ramularia cyclaminicola*, *Septoria cyclaminis* and *Thielaviopsis basicola*, can successfully settle on roots, corms and leaves of *Cyclamen* plants. One explanation for this apparent insensitivity could be that these fungi produce one or several enzymes which can degrade and thereby inactivate the saponin. Such enzymatic activity would allow fungal development on previously resistant tissues because of the partial or complete inactivation of cyclamin. To test this hypothesis mycelial discs of a number of fungal species were incubated in a medium containing 1.500 μg cyclamin/ml. After four days of incubation the

Table 5

Inactivation of cyclamin by fungal species, growing on a liquid medium with 1.500 μg cyclamin/ml

Fungal species	Growth	μg cyclamin[a]
Pathogens on *Cyclamen*:		
Botrytis cinerea	+++	100
Cryptocline cyclaminis	++	300
Ramularia cyclaminicola	±	1500
Septoria cyclaminis	+++	1500
Pathogens on other saponin-containing plants:		
Colletotrichum hedericola	++	1500
Colletotrichum trichellum	++	1500
Pestalotia microspora	+++	300
Colletotrichum atramentarium	+	1500
Rhizoctonia solani	0	1500
Septoria lycopersici	±	1500
Pathogens on saponin-free plants:		
Fusarium nivale	+++	1500
Alternaria brassicae	±	1500
Saprophytes:		
Aspergillus niger	±	1500
Aspergillus ochraceus "Ivy"	+	1500
Rhizopus nigricans	+++	1500

[a] Cyclamin retained in the medium after 4 days of incubation with the different fungal species.

0 = no growth, ± very limited growth, + = some growth, ++ = good growth, +++ = excellent growth (the volume of mycelium being almost the same as that of the medium)

fungal growth was checked and the cyclamin retained in the medium was determined (Table 5).

None of the saprophytes, pathogens of saponin containing plants other than *Cyclamen* did inactivate the cyclamin, with the exception of *Pestalotia microspora*, which is a weak pathogen on a variegation of English ivy *(Hedera helix)*. The reaction of fungi pathogenic to *Cyclamen* was not uniform. *B. cinerea* and *C. cyclaminis* did partially inactivate the saponin whereas *R. cyclaminicola* and *S. cyclaminis* did not.

With all fungi there was no correlation between growth rate and saponin inactivation. Although they did not inactivate the saponin, some fungal species like *Rhizopus nigricans* and *Fusarium nivale* grew remarkably well despite the relatively high cyclamin content of the medium. No explanation can be given at present

for this phenomenon. However, of what nature the protective mechanism of these fungi may be, it has to be different than that of *B. cinerea*.

Since *B. cinerea* did inactivate cyclamin best of all fungi it was investigated whether this was due to the production of an adaptive enzyme. The fungus was grown on a glucose-yeast extract medium which contained 1.500 μg cyclamin/ml. After four days of incubation on a rotary shaker, when the development of fungal mycelium had reached the end of the growth phase, the cyclamin in the medium

Table 6

The lack of cyclamin inactivating enzymatic activity in the culture filtrate, and both the particulate and supernatant fraction of homogenates from *Botrytis cinerea* mycelium

Material	Treatment	μg/cyclamin/ml after		
		1	2	15 hrs
Unused medium	—	1500	1500	1500
Culture filtrate	boiled	1500	1500	1500
	not boiled	1500	1500	1500
Particulate fraction	boiled	1500	1500	1500
	not boiled	1500	1500	?
Supernatant fraction	boiled	1500	1500	1500
	not boiled	1500	1500	1500

had decreased from 1.500 to 150 μg/ml. Using this material it was tested whether there was any cyclamin inactivating enzymatic activity in the culture filtrate and both the particulate and supernatant fraction of homogenates obtained from fungal mycelium. These fractions were incubated with cyclamin at a concentration of 1.500 μg/ml and after 1,2 and 15 hours the cyclamin retained in the incubation medium was determined. According to the results (Table 6) no enzymatic degradation had occurred in any of the fractions. The particulate fraction gave inconsistent results after 15 hours of incubation with cyclamin values between 200 and 1.500 μg/ml. Since grinding with sand, a standard procedure to obtain homogenates from fungal mycelium, does not destroy all cells new mycelium developed in this fraction. It is to this new mycelium that the inconsistency after 15 hours incubation must be attributed.

The most important fungal pathogen on cultivated *Cyclamen* plants is the grey mold, *B. cinerea*. The infection usually starts on the stems and later progresses towards the leaves and flowers. In view of the ability of *B. cinerea* to inactivate cyclamin it was interesting to study the development of this fungus on different plant parts in relation to their saponin content. Artificial inoculations on wounded stems resulted in about 50% positive infections, the recovery of the fungus from

Table 7

Susceptibility of stems and leaves of *C. persicum* (cultivated type) to artificial inoculation with *Botrytis cinerea* and recovery of the fungus one week after inoculation

Treatment	No. of inoculations or wounds	Infections %	% Recovery of B. cinerea
Wounded but not inoculated with *B. cinerea*			
Stem	50	2	4
	50	0	0
Leaf	27	0	0
	27	0	0
Wounded but inoculated with *B. cinerea*			
Stem	77	46	—
	100	61	74
	100	46	57
Leaf	71	0	—
	72	0	0
	87	0	0

the sites of inoculation after one week was about 10% higher (Table 7). Inoculations on wounded leaf surfaces were negative, the fungus neither grew nor was its recovery possible one week after inoculation. Most probably the fungus was confronted with too high a saponin concentration at once, there was no time to allow the build up of the inactivation mechanism, hence the fungus was killed.

Considering all the available informations the development of *B. cinerea* on *Cyclamen* could be as follows. The fungus first invades the stems; their low saponin content does permit its unhindered growth. The cyclamin in the stem, although present only at a low concentration, induces the cyclamin inactivating mechanism which may or may not move towards the leaf in advance of the fungal mycelium. This mechanism enables the fungus to overcome the saponin gradient which extends from the stem base to the leaf and thus allows the final settlement on the leaf which had been weakened before by the partial or complete block of the vascular system due to the macerating enzymes of the fungus.

Cyclamin as a resistance factor

The roots, corms and leaves of *C. persicum* contain cyclamin at concentrations 10 to 1.000 times the LD_{100} required for all fungi tested, therefore, this saponin must be regarded as a potent antifungal barrier. In contrast to the other plant parts, the stems are almost devoid of cyclamin and are thus liable to become primary sites of infections.

There is, however, a number of fungi which can successfully settle on *Cyclamen* parts despite their high saponin content. One way of overcoming this fungi-

cidal barrier would be the production of saponin degrading and thereby inactivating enzymes, as described by TURNER (1960) and ARNESON and DURBIN (1967, 1968). The experiments conducted with several fungal pathogens of *Cyclamen* did not substantiate this hypothesis. Two of the pathogens, *Ramularia cyclaminicola* and *Septoria cyclaminis*, could not inactivate cyclamin at all. *Botrytis cinerea*, which did so, had no enzymatic activity in the culture filtrate, and both the particulate and supernatant fraction of mycelium homogenates, although the cyclamin concentration in the medium had decreased from 1.500 to 150 μg/ml. An enzyme participation in this phenomenon can not be entirely excluded at present but only future experiments on a strictly chemical basis could elucidate the nature of this mechanism.

Not all the parts of saponin containing plants have the fungicidal barrier. Stems for example can be almost devoid of saponins as is the case with *Cyclamen* and tomatoes (KERN, 1952, SANDER, 1955). Thus, there is a way by which fungi can by-pass the saponin barrier and still develop on plants which in most of their other parts have a respectable defense in their high saponin content. Besides, the example of *B. cinerea* on *Cyclamen* other fungi such as *Didymella lycopersici* and *Sclerotium rolfsii*, both parasitizing on tomato stems, could fall into this category of host–parasite relation.

Interesting in respect to saponin depending host–parasite interactions is the report by SIJPESTEIJN and VAN DIJKMAN (1971) on the biochemical basis for the defense reaction of tomato to *Cladosporium fulvum*. Non-pathogenic races of this fungus destroyed the selective permeability of the host cells. As a consequence, a hypersensitivity reaction occurred but at the same time considerable amounts of tomatine must have been released and come into contact with the fungal mycelium, especially the growing tips. This tomatine, having a strong fungicidal activity, could well account for the killing of the fungus. On the other hand, pathogenic races of this fungus did not produce membranolytic substances, therefore, there could have been no tomatine release. No hypersensitive reaction was observed. This finding indicates the complexity of host–parasite relations. When fungal pathogens can overcome the defense mechanism it will not be on a one step basis, for example saponin – enzymatic inactivation, but rather as a consequence of a chain of reactions.

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Isolation and Some Properties of Biologically Active Substances from Apple Bark*

By

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Biologically active components have been isolated from apple bark and partially characterized. These substances possess activity when tested on 3 species of *Phytophthora* and *Endothia parasitica*, as reflected by a stimulation or inhibition in growth of the fungus on MM or LBA media containing these fractions. Two substances which stimulate the *in vitro* growth of *P. cactorum* have been found in a tolerant variety, Starking, while one with similar properties occurs in Grimes, a highly susceptible variety. Both Grimes and Starking contain 2 groups of substances which are inhibitory to the growth of the fungus, one of which can be isolated only when the extraction media contains Na_2SO_3. Their quantitative and proportional presence as well as their susceptibility to oxidative inactivation are believed to be associated with the host resistance-mechanisms.

Collar rot caused by *Phytophthora cactorum* is a serious disease of apple trees in many fruit growing areas. Certain apple varieties such as Grimes Golden are extremely susceptible while others, including Antonovka and Starking, possess high levels of resistance. The susceptibility of the host, however, varies with the age of the tree and season, suggesting a probable role of auxins (SCHWINN, 1965) in disease initiation and development. Current season's growth is completely susceptible until early August while one year-old growth shows 3 seasonal peaks of susceptibility (BRAUN and NIENHAUS 1959). Under Missouri conditions the first peak of susceptibility occurs during blossoming, the second in late June and the third 30 to 40 days later.

Early investigations by BORECKI showed that the greatest fungal growth develops in the inner phloem-cambium tissue of apple bark. Lyophilized samples

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of this tissue when extracted with 0.01 *N* NaOH and passed through a Sephadex G-25 column yield 4 groups of substances with absorbance at 254 nm (BORECKI et al. 1970). Materials within the second peak stimulated the in vitro growth of *P. cactorum* while those in the third peak are inhibitory. These substances are heat labile and lose their activity upon dialysis. When Na_2SO_3 is added to the extraction media as a reducing agent an additional substance with strong inhibitory properties is found (GATES and CIMANOWSKY).

Materials and Methods

One-year-old wood was collected and the outer bark removed. The inner bark was scrapped, immediately frozen (with liquid nitrogen) and lyophilized. The dried tissue was pulverized in a Spex mixer mill and stored in a closed container at −20 C until used. For extraction, 15 g of the lyophylized tissue was suspended in 6 vol of 0.01 *N* NaOH plus 2% (w/v) Na_2SO_3 and refrigerated for 45 min. The supernatant was removed by filtration through a cheesecloth on a Buchner funnel under partial vacuum. Cellular debris was removed by centrifugation at 17,000 rpm in a Servall RC-2 centrifuge for 10 min at 3°C. The supernatant was decanted, brought to 75 ml vol with distilled water and stored under refrigeration if not used immediately.

The supernatant was passed through a 42 × 5 cm column of Sephadex G-25 previously hydrated in 0.02 *M* phosphate buffer, pH 6.7. A filter paper disc on the upper surface serves as the column stabilizer. Before use, the column was calibrated with a blue dextran 2000 dye, made 1% in buffer (w/v) to determine the void volume of the column. Subsequently, a 50 ml aliquot of the extract was applied to the surface of the Sephadex column and allowed to diffuse into the gel. Phosphate buffer (pH 6.7) was then introduced and the column was attached to a buffer reservoir. The flow rate was adjusted to about 2 ml/min and 20 ml fractions were collected. The effluent was monitored at 254 nm by an ISCO UV analyzer. Fractions exhibiting similar absorption (350 − 220 nm) are combined, sterilized by micropore filtration and stored under refrigeration until used. The samples are designated as follows: Grimes XII (113 − 130) indicated the twelfth sample from Grimes tissue containing fractions 113 through 130 ml which showed the same absorption spectra. Starking SS XI (101 − 120) is the eleventh sample of the Starking extract, the SS indicates it had been protected from oxidation with Na_2SO_3.

P. cactorum 15, a highly pathogenic culture isolated from diseased apple root near New Franklin, Missouri, is routinely used as a test organism. For bioassay a weak mineral media (MM) composed of 24 g of sucrose, 4 g of $NaNO_3$, 4 g of KH_2PO_4 m, 1 g of KCl, 0.6 g of $MgSO_4$ and 20 g of agar per litre of distilled water; or difco lima bean agar (LBA) was used. Both media used for bioassay contain 50% of the required water, are steam sterilized and diluted with equal amounts of purified extracts in sterile 5 cm petri dishes. These plates are inoculated

with a 3 mm disc of the fungus removed from an actively growing culture. Differences in the diameter of colonies between those growing on supplemented and control media after 2 1/2 days at 26°C are used as a measure of biological activity.

Results

Fig. 1 shows a typical 254 nm profile of a $NaOH - Na_2SO_3$ extract of lyophilized inner bark from one-year-old wood of Starking cut in December, 1968. This same general pattern is also found on the Grimes extract. The areas of biological activity were determined by combining individual fractions with similar UV transmission patterns and assayed for stimulation or inhibition of growth.

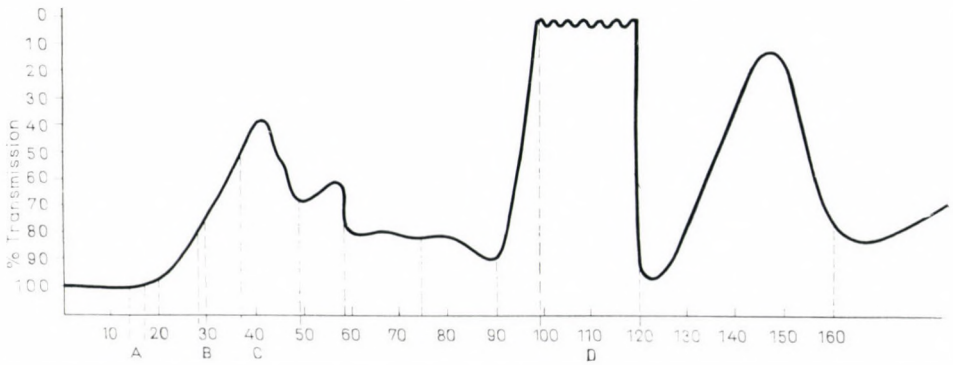

Fig. 1. Fraction profile of eluates from $NaOH - Na_2SO_3$ extracts of lyophilized inner phloem-cambium tissue. Current season's growth of starking cut in December, 1968. From left to right: A = Stimulator II, B = Absolute inhibitor, C = Stimulator, and D = Inhibitor

In both Starking and Starking SS extracts a group of substances come off in the first 300 to 500 ml fraction of eluate (Stimulator II) and are referred to as Starking I and II (18 – 28) and Starking SS II (14 – 17), respectively. These fractions may or may not contain similar substances since the former is quite stimulatory to the *in vitro* growth of the fungus while the latter is only slightly stimulatory. The UV absorption of the Starking SS II extract resembles that of nucleic acids, having a maximum at 260 nm and minimum at 230 nm while the Starking I and II extract has a rather indistinct pattern. For both Starking and Grimes, substances come off the column in the first 500 to 1000 ml volume of eluates and are referred to as Grimes V (36 – 55) and Starking II (29 – 39). The substances possess similar absorbance at 470 nm and apparently are present in approximately equal concentrations. The Grimes fraction stimulates growth of *P. cactorum* while that from Starking causes some stimulation on MM but shows a slight inhibitory effect when added to LBA. If Na_2SO_3 is added to the extraction media similar substances are

found in the Starking SS V (29–38) fraction. Their absorption spectra are similar to the NaOH extracts but they lack the characteristic peak at 470 nm. They occur in the Grimes SS extracts and contain 2 to 4 times as much protein as those appearing in the NaOH extracts. Substances in this sample completely inhibit the growth of *P. cactorum* on LBA but only slightly on MM and are considered as an absolute inhibitor. This sample gives a positive Kjeldahl reaction and contains at least 5

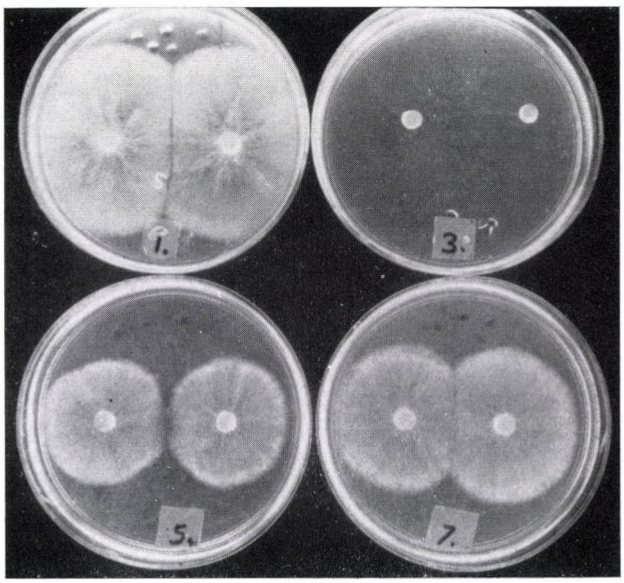

Fig. 2. Growth of *P. cactorum* on LBA. Lower right (7) control (LB); Lower left (5) LBA and inhibitor; Upper left (1) LBA and stimulator; Upper right (3) LBA and absolute inhibitor

identifiable amino acids. The substances within this fraction do not elicit separate peaks at 254 nm and are included in the fraction previously considered as stimulatory (BORECKI et al., 1970).

Another fraction comes off in the 100–130 tube fractions, regardless of the extraction media. It is inhibitory to the growth of the fungus with the degree of inhibition being quantitatively proportional to the amount of substances reacting positively with the Lowry reagents. These active ingredients appear in samples Grimes XII (113–130), Grimes SS XI (102–124), Starking IX (75–84) and Starking SS XI (101–120) with the greater amounts occurring in the extracts protected from oxidation. Starking IX is more inhibitory than Grimes XII while the Grimes SS extract is more inhibitory.

Biological tests of these fractions are summarized in Table 1. Other studies involved the testing of the Grimes active fractions against *P. cinnamomi* and *P.*

Table 1

Biological activity of bark extracts from lyophilized current season's growth of Grimes and Starking apple. Wood cut in December, 1968[1]

Effect	Media	Variety	Sample fraction	Clonial growth		
				Total[2]	(mm) Corrected	% Control
Inhibitory	LBA	Grimes	V (36—55)	24.6	26.7	126.7
			XII (113—136)	19.8	—15.8	84.2
			control	21.7		100
			SS II (18—28)	30.6	16.6	116.6
			SS IV (34—42)	0.0	—00.1	9.9
			SS XI (102—124)	20.4	—44.7	55.3
			control	25.4		100
		Starking	I & II (18—28)	25.8	—11.8	118.8
			III (29—39)	21.9	7.0	93.0
			IX (75—84)	17.3	—25.7	74.3
			control	23.3		100
			SS II (14—17)	32.5	4.4	104.4
			SS V (29—38)	0.0	—100	0.0
			SS XI (101—120)	23.7	—38.2	61.8
			control	29.3		100
Stimulatory		Grimes	V (36—53)	14.6	156.1	256.1
			XII (113—130)	8.2	0.0	100
			control	8.2		100
			SS II (18—28)	23.5	75.2	175.2
			SS IV (34—42)	19.4	54.5	154.5
			SS XI (102—124)	10.1	—38.0	62.0
			control	12.1		100
		Starking	I & II (18—28)	18.3	124.3	224.1
			III (29—39)	10.1	24.3	124.3
			IX (75—84)	8.2	—4.7	95.3
			control	8.6		100
			SS II (14—17)	13.4	1.9	101.9
			SS V (29—38)	11.5	—10.2	89.8
			SS XI (101—120)	10.4	—37.5	62.5
			control	12.8		100

[1] Data from GATES and CIMANOWSKI.
[2] Each estimate represents a mean of at least 6 individual measurements.

citricola as well as the bark parasite, *Endothia parasitica*. The growth of all 3 fungi on the media was similar to that of *P. cactorum*, suggesting a possible specificity for pythiaceous root rotting fungi and bark parasites but more extensive investigations are needed to clarify this point. When these substances were tested against the plant pathogen, *Erwinia amylovora*, they were found to be inactive. These observations (as well as occasional bacterial contamination in bioassays) indicate that these substances are not biologically active against bacteria.

Discussion

Our data demonstrate the presence of endogenous host substances in the inner bark of apple which can stimulate or inhibit the *in vitro* growth of *P. cactorum*. They have been partially characterized and are present in quantities and proportions which appear to parallel the host's natural resistance. Biological tests with other species of *Phytophthora* and the bark parasite, *Endothia parasitica*, suggest a specificity for fungal bark pathogens but further investigations are needed before any definitive conclusions can be drawn.

Qualitative as well as quantitative differences apparently are associated with varietal resistance. Starking, a tolerant variety, possesses substances which stimulate the growth of the fungus. These compounds have UV spectra similar to those of nucleic acids and are affected by oxidative enzymatic action but are absent in the susceptible Grimes. Both varieties also contain substances which inhibit fungal growth, but these are quantitatively greater in Starking. The precise relationship of these compounds to varietal resistance, however, must await additional studies involving the influence of other factors, including auxins, season and age of the host.

Other inhibiting substances also are found in both varieties. They can be isolated only when the extracting media contains Na_2SO_3 to protect against oxidation and appear to be proteinaceous in nature. If these substances play a role in this host/parasite association and are susceptible to *in vivo* oxidation as *in vitro* tests indicate, the ability of the fungus to produce a sufficiently active enzyme may be an important mechanism for establishing successful infection. This possibility is suggested by the observations of BORECKI (unpublished) who noted that the browning of host tissue always preceded fungal invasion. Additional evidence is found in the Grimes and Starking extracts. If the Grimes extract is not protected from oxidation its activity is less than that of Starking but when Na_2SO_3 is added to the extraction media the inhibition is greater.

Conversely, the ability of the host to resist fungal enzymatic action would prevent this oxidative inactivation. Other studies in our laboratory and elsewhere (SCHWINN, 1965) have shown that the addition of indole acetic acid (IAA) increases host susceptibility. Possibly IAA may induce fungal enzyme activity and in this manner decrease host resistance. Additionally, the 3 seasonal peaks in host susceptibility (BRAUN and NIENHAUS, 1959) occurring during periods associated with blossoming, flower bud differentiation and the onset of dormancy provide circumstantial evidence of *in vivo* role of host auxins in susceptibility. The identity of specific auxins and their role in host susceptibility, however, has not been established.

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Bacteriostatic Compounds from Bean Leaves

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Trifoliate leaves from different varieties and strains of *Phaseolus vulgaris*, which showed different degrees of tolerance towards *Xanthomonas phaseoli* var. *fuscans*, were analysed for bacteriostatic compounds in order to explain different levels of bacterial multiplication within the leaf. By application of the methods of PIERRE and BATEMAN (1967) we did not find detectable levels of phytoalexins in leaf homogenates of healthy and infected susceptible and resistant plants after extraction with petrol ether and ethyl acetate. Testing the intercellular fluid, according to KLEMENT (1965) and STALL and COOK (1968), there was a clear evidence for bacteriostatic compounds in resistant varieties in contrast to susceptible varieties. (The leaves were infiltrated with phosphate buffer and centrifuged on a sieve. The fluid was steril filtrated, inoculated with bacteria and the multiplication analysed 20, 40 and 60 hours after incubation at 25°C). Leaves treated with water by means of a spray gun showed the highest concentration of bacteriostatic compounds 1—3 days after treatment, while leaves treated with a bacterial suspension by means of a spray gun contained comparable low amounts of bacteriostatic compounds. Untreated leaves contained no bacteriostatic compounds. Also 7 days after treatment no bacteriostatic compounds were detectable. High temperatures after spray gun treatment promoted appearance of bacteriostatic compounds. Bacteriostatic compounds were not detectable if plants were kept at 100% humidity in the dark after treatment. The bacteriostatic compounds were very active against *Xanthomonas phaseoli* var. *fuscans*, *Pseudomonas phaseolicola*, *Pseudomonas syringae* and *Xanthomonas campestris* but showed only low inhibition of *Pseudomonas tabaci* and *Escherichia coli*. Some of the obtained intercellular fluids showed total inhibition of multiplication of *Pseudomonas phaseolicola* if diluted 1 : 36 with intercellular fluid containing no bacteriostatic compounds. Autoclaving did not inactivate the bacteriostatic compounds.

The two bacterial bean diseases of economic significance in Germany are halo-blight, caused by *Pseudomonas phaseolicola* [15], and fuscous blight, caused by *Xanthomonas phaseoli* var. *fuscans* [2, 17]. The degree of susceptibility of bean varieties towards these two bacterial diseases varies. In cooperation with a bean breeding company we detected some strains of *Phaseolus vulgaris* which showed rather high tolerance towards fuscous blight [1]. When such bean leaves are artificially inoculated with *Xanthomonas phaseoli* var. *fuscans* they show nearly no symptoms. On the other hand, susceptible cultivars develop greasy looking translucent spots, which may cover the whole leaf with susceptible varieties. When such leaves are placed on photo-paper and illuminated, the developed paper shows

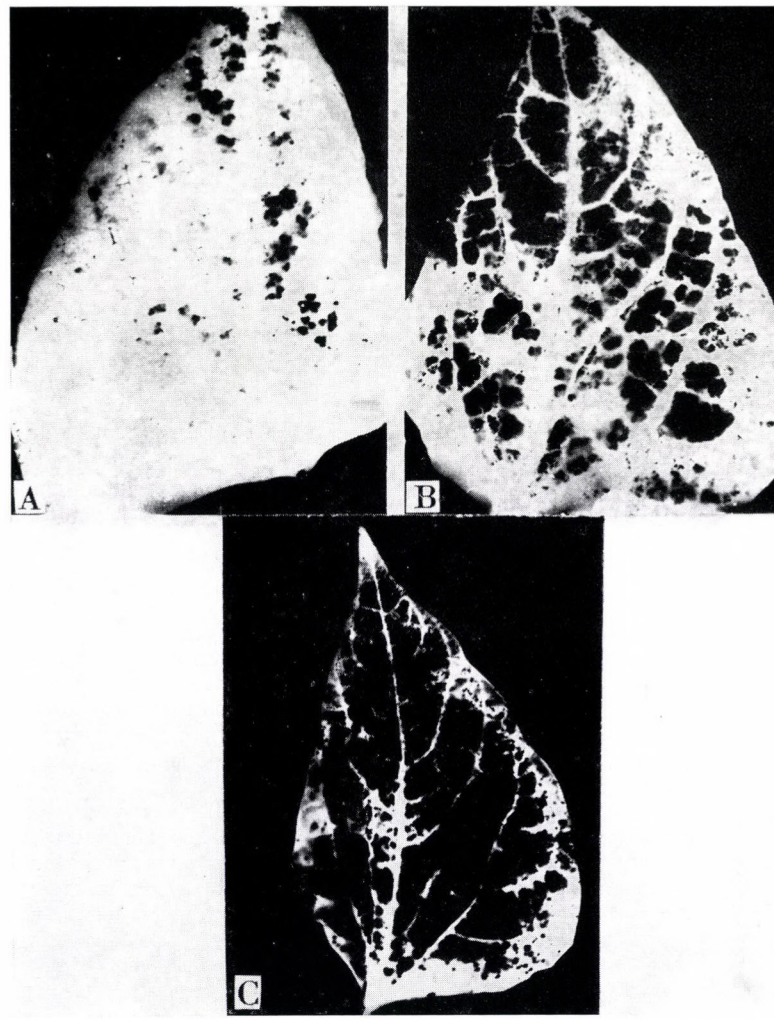

Fig. 1. Leaves of susceptible and weakly resistant bean varieties after infection with *Xanthomonas phaseoli* var. *fuscans*. A 09: Weakly resistant; B 181: Susceptible; C: Red Kidney: Very susceptible

dark areas under the translucent spots. This is demonstrated by Figure 1. Infection of the very susceptible variety Red Kidney resulted in large translucent areas, which covered nearly the whole leaf. Another susceptible variety, P. I. 181 954, showed also many translucent spots, but to a lower extent than Red Kidney. Even less translucent spots were observed with the moderate susceptible strain 09. Figure 2 shows pictures of two resistant strains. The highly tolerant 02 did not

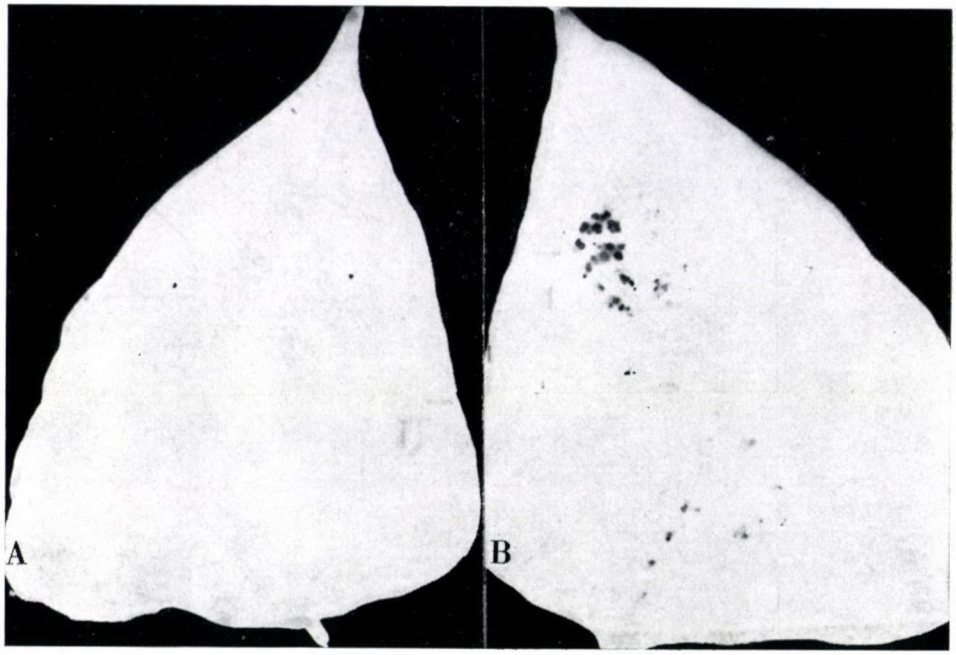

Fig. 2. Leaves of resistant bean varieties after infection with *Xanthomonas phaseoli* var. *fuscans*. A 02: Resistant; B 017: Resistant

develop visible translucent spots, and only a few translucent spots could be demonstrated on 017. The estimation of bacterial propagation within the leaves gave similar differences between these five bean varieties [1].

We assumed that the mechanism of resistance towards *Xanthomonas phaseoli* var. *fuscans* is principally the same with different bean varieties. In this case different intensities of the resistance inducing process should result in different degrees of susceptibility. We tried to prove this hypothesis by looking for bacteriostatic compounds in bean leaves with and without infection.

Two different methods were applied: 1) Leaf homogenates were extracted with organic solvents and the extracts tested against fungi and bacteria; 2) the intercellular fluid was rinsed out of the leaves without injury and tested against bacteria.

1) Extracts from leaf homogenates were prepared according to PIERRE and BATEMAN [14], who demonstrated two fungistatic compounds in bean hypocotyls after infection with *Rhizoctonia solani* (homogenization in ethanol, centrifugation, lyophilization, solution in water extracted once with petrol ether, then with ethyl acetate). None of the obtained extracts from resistant and susceptible healthy and infected leaves did show any bacteriostatic or fungistatic activity as well as no specific absorption in the UV, as observed by PIERRE and BATEMAN.

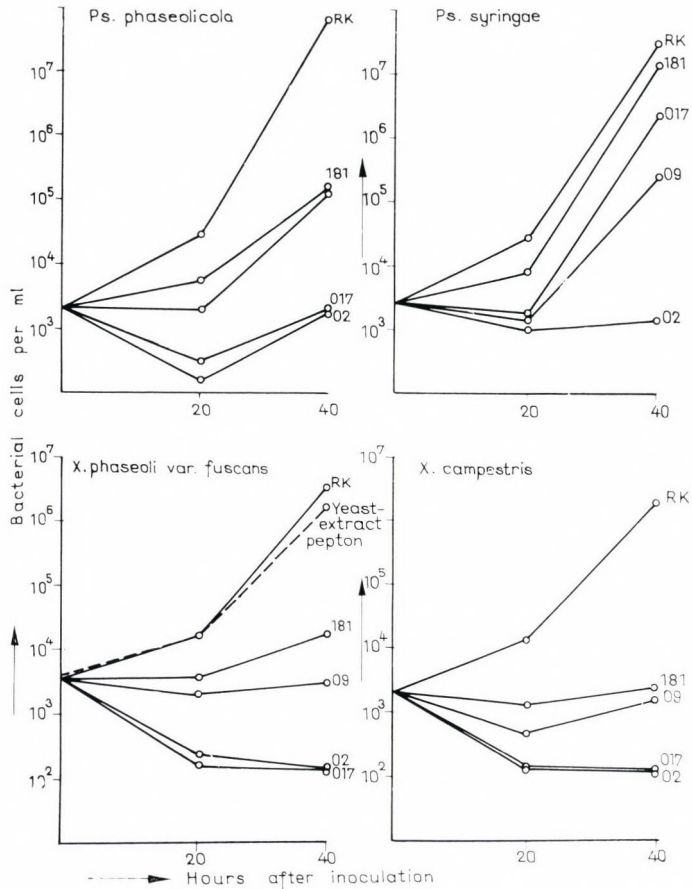

Fig. 3. Growth of phytopathogenic bacteria in the intercellular fluid of bean leaves 3 days after spray gun treatment with water

2) The intercellular fluid was obtained according to KLEMENT (10) and STALL and COOK [18]. Leaves were infiltrated with 0.034 *M* phosphate buffer of pH 7.0 in 0.85% NaCl. The phosphate buffer was centrifuged out of the leaves together with the intercellular fluid. After sterile filtration the intercellular fluid was inoculated with bacteria and incubated in a shaker at 25°C. The bacterial multiplication was estimated in intervals of 20, 40, and 60 hours after inoculation. The bean leaves, used for these experiments, were artificially inoculated with a bacterial suspension by means of a spray gun [1]. For comparison leaves were treated with water only by a spray gun.

In contradiction to our expectations a strong inhibition of bacterial multiplication was observed in water treated leaves but not in infected leaves (Fig. 3),

when the intercellular fluid was obtained 3 days after treatment. The intercellular fluid from resistant leaves (02, 017) showed the strongest inhibition, whereas the intercellular fluid from the very susceptible variety Red Kidney showed no inhibition at all, when compared with yeast extract peptone. The other susceptible variety, P. I. 181 954, showed some inhibition, and the weakly resistant strain 09 showed an inhibition between 181 and 017/02.

Table 1

Bacterial cells of *Xanthomonas phaseoli* var. *fuscans* per cm^2 leaf

Bean variety	Reaction	Days after infection	
		2	3
Red Kidney	very susceptible	61.0×10^5	520×10^5
181 954	susceptible	32.0×10^5	350×10^5
09	weakly susceptible	7.1×10^5	25×10^5
017	resistant	7.2×10^5	11×10^5
02	resistant	6.5×10^5	9×10^5

Because of this clear correlation between content of bacteriostatic compounds and resistance, we assume that the observed bacteriostatic effects in water-treated leaves play a role in resistance after infection, too.

The bacteriostatic effect was observed towards three bacterial pathogens of bean *(Ps. phaseolicola, Ps. syringae, X. phaseoli* var. *fuscans)* as well as against another phytopathogenic bacterium *(X. campestris)* (Fig. 3). However, another phytopathogenic bacterium, *Ps. tabaci*, as well as *E. coli* were not inhibited by the extracts (Fig. 4.).

When the intercellular fluid was taken 7 days after treatment instead of 3 days, there was nearly no bacteriostatic effect observable (Fig. 5). Only 02 showed an inhibition of *X. phaseoli* var. *fuscans*. Untreated leaves did never show comparable bacteriostatic effects. Obviously, bacteriostatic compounds appear in resistant varieties shortly after water treatment by the spray gun and then disappear again.

If we assume that the demonstrated bacteriostatic compounds play a role in resistance, the open question remains, why we did not detect them in infected resistant leaves. Considering this question we should regard that the so-called resistant or tolerant varieties do not exclude any bacterial multiplication after artificial infection, as shown in Table 1. Although there is an about 50-fold higher multiplication of *Xanthomonas phaseoli* var. *fuscans* in the susceptible variety compared with the resistant one, also in the resistant variety a bacterial concentration of 10^6 cells per cm^2 was reached. We conclude, therefore, that the bacteria are able to inhibit formation of bacteriostatic compounds to some extent or to enhance their metabolization. The equilibrium between concentration of bacterio-

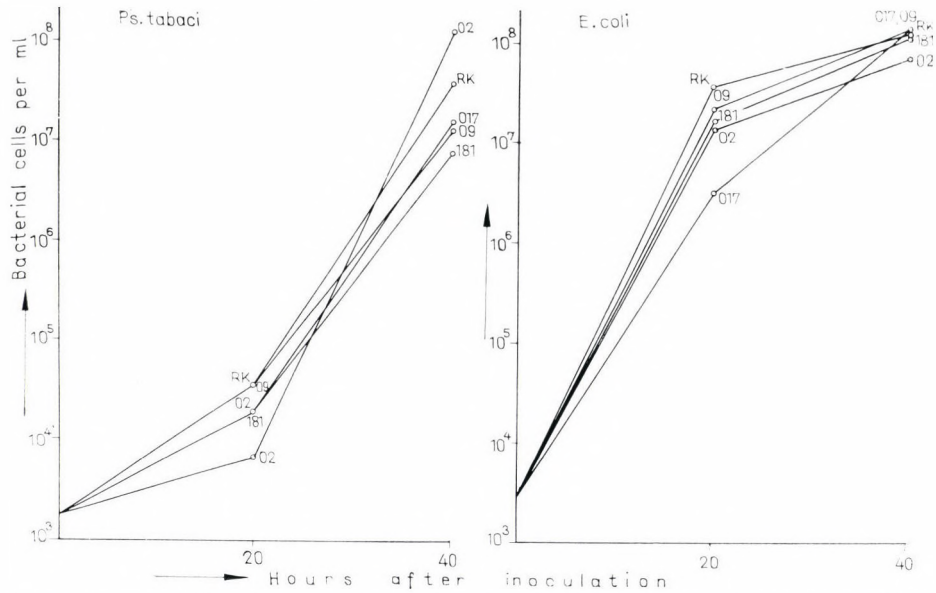

Fig. 4. Growth of *Pseudomonas tabaci* and *Escherichia coli* in the intercellular fluid of bean leaves 3 days after spray gun treatment with water

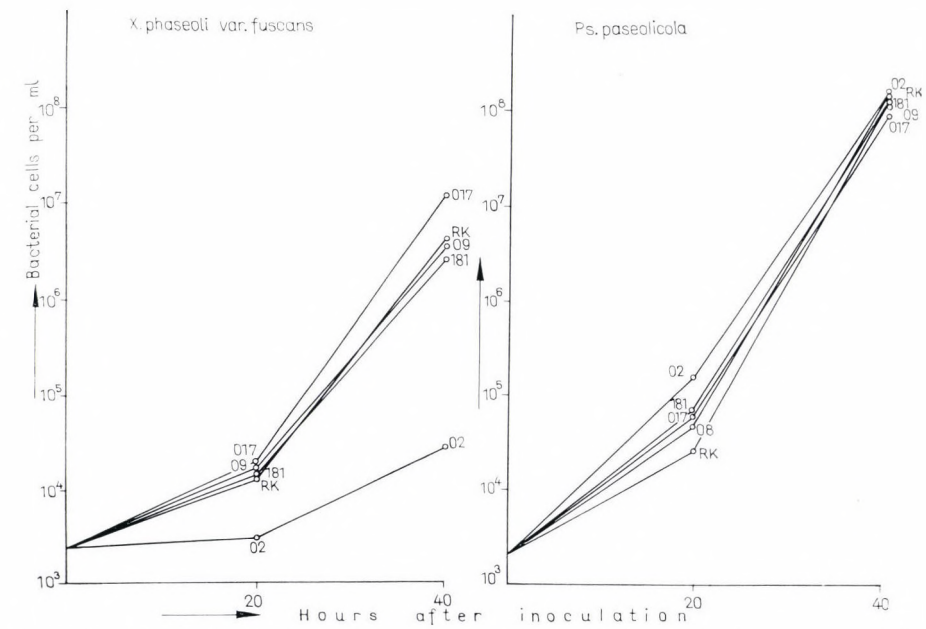

Fig. 5. Growth of phytopathogenic bacteria in the intercellular fluid of bean leaves 7 days after spray gun treatment with water

static compounds on one side and activity of bacteria on the other side may be different for each host–parasite combination.

The first experiments were carried out under green-house conditions, where we did not find bacteriostatic compounds in every experiment. Therefore, we studied the factors which influenced their formation by testing the very resistant cultivar 017 (Table 2). 24 hours after treatment we observed a very strong inhibition

Table 2

Growth of *Pseudomonas phaseolicola* in the intercellular fluid of not infected resistant bean leaves (strain 017) 40 hours after inoculation

	Temperat. °C	Illumination	Humidity %	Bacterial cells per ml	
				Leaves harvested	
				24 hours after treatment	38
Greenhouse	16–26	light	40–80	3.0×10^{7}	$<1\times10^{1}$
Greenhouse	15–25	dark	100	2.3×10^{8}	2.6×10^{8}
Growth chamber	20	light	50–60	1.6×10^{3}	3.0×10^{8}
Growth chamber	30	light	80–90	$<1\times10^{1}$	3.6×10^{4}

after incubation in a growth chamber of 30°C, less inhibition at 20°C, very low inhibition in green-house, and no inhibition if incubated in 100% humidity and darkness. 38 hours after treatment incubation at 30°C growth chamber gave clear inhibition, whereas incubation at 20°C did not. Also incubation at darkness in 100% humidity gave no inhibition, but a very high inhibition was obtained after incubation in the green-house. The varying conditions in the green-house do not allow a clear evaluation of this last observation. However, it could be demonstrated by these experiments, that the formation of bacteriostatic compounds is favoured by high temperatures about 30°C, and that bacteriostatic compounds are not formed in darkness at 100% humidity.

In order to study the potency of the bacteriostatic compounds, dilution experiments were carried out. Intercellular fluids with bacteriostatic properties were mixed with intercellular fluids which showed no inhibition of bacterial multiplication. Some extracts gave complete inhibition of bacterial growth when diluted 1 : 36. If we consider that our preparations are already dilutions of the true intercellular fluid, since they are obtained by rinsing the intercellular spaces with phosphate buffer, it is evident that the bacteriostatic compounds occur in a rather high biological concentration within the intercellular spaces. The dilution experiments did also confirm that we are dealing with bacteriostatic compounds, and not slow growth of the bacteria because of lack of nutrients.

The chemical characterization of these compounds has just started. They are stable towards autoclaving.

Discussion

Antibacterial compounds in higher plants have been described frequently [6, 7] and designated as "phytoncides" by russian workers [19, 20]. However, it has been seldom established that such compounds are responsible for resistance. In our case, it is possible that the detected bacteriostatic compounds are decisive during the very early stages of disease, which are relatively short with some bacterial infections [11, 12]. Because of the high concentration of these compounds, as demonstrated by the dilution experiments, we suggest that they may inhibit bacterial growth to a varying extent at a very early infection stage. It is also possible that changes in host metabolism which are favourable or necessary for bacterial propagation [16], are influenced by these compounds.

Although the bacteriostatic compounds were not detected in infected leaves, they do not represent typical axenic, that is preformed inhibitory compounds [8]. Preformed bacteriostatic compounds have, for instance, been demonstrated in the gynaeceum of *Primula obconica* without presence of microorganisms [9]. In our case bacteriostatic compounds did appear after spray gun treatment with water, only. Although we tried to prevent any injury by this treatment, we assume that the leaves were exposed to a certain stress, which – may be in a specific way – resulted in the temporary appearance of bacteriostatic compounds.

Applying the same methods, STALL and COOK [18] found inhibiting substances towards *Xanthomonas vesicatoria* in the intercellular fluid of hypersensitive varieties of *Capsicum annuum* 16 hrs after infection. Susceptible plants did not show such compounds. Further investigations with bacterial infected leaves have to elucidate whether resistant cultivars do liberate bacteriostatic compounds within short intervals after infection.

OMER and WOOD [13] recorded inhibitory substances towards *Pseudomonas phaseolicola* in extracts from leaf homogenates with the strongest inhibition 3 days after infection. In extracts from healthy leaves the bacteria propagated 3 times faster than in extracts from infected leaves. The bacteriostatic compounds obtained under our conditions showed much stronger inhibitions. Obviously, the intercellular fluid allows easier detection of bacteriostatic compounds. The mixing with cell constituents in the case of homogenates may inactivate or neutralize bacteriostatic compounds.

We did not find any bacteriostatic effect in extracts from leaf homogenates. However, our results are not completely comparable with those of OMER and WOOD [13], since we have used ethyl acetate and petrol ether extracts. By application of this method PIERRE and BATEMAN [14] demonstrated the presence of the phytoalexin phaseollin and another similar compound in bean hypocotyls after infection with *Rhizoctonia solani*. The fact that we did not find these phytoalexins in fuscous blight infected bean leaves was not surprising, since: a) the closely related phytoalexin pisatin from peas does not appear after bacterial infection [5] and b) since pisatin shows only low inhibition of bacteria as compared to fungi [3].

It appears, therefore, as if the detected bacteriostatic compounds from the intercellular fluid differ from the classical phytoalexins, as reviewed by CRUICKSHANK (4). Similar to phytoalexins, these compounds show only low specificity. With one exception *(Ps. tabaci)* all analysed phytopathogenic bacteria were inhibited.

Further investigations have to deal with origin, role and chemical nature of these compounds.

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The Hypersensitive Reaction of Plants to Bacterial Infections

By

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The hypersensitive reaction (HR) is a general response of plants against infection in all incompatible host-bacterium relation. It was possible to distinguish three phases during the development of the HR. The induction time (3—4 hr) is independent from host, but it depends on the metabolic activity of bacteria. The latent period is independent from the presence of bacterium. During this period no symptoms appear, but some physiological and biochemical changes occur. After the latent period tissue collapse is rapid, because the permeability of the host cell membrane has changed.

The hypersensitive reaction (HR) is a general response of plants against infection. HR induced by phytopathogenic bacteria was clearly established in the sixties (KLEMENT, 1963; KLEMENT, FARKAS and LOVREKOVICH, 1964). In our laboratory we have investigated different host—parasite relationships in relation to HR. Table 1 shows the types of symptoms and the appearance of HR in the various host—pathogen combinations.

Table 1

Types of symptoms and the appearance of the hypersensitive reaction in different plant—pathogen combinations

Combinations	HR	Typical disease symptom
Virulent bacteria—sensitive host plant	—	+
Avirulent bacteria—normally sensitive host	+	—
Normally virulent bacteria—resistant host	+	—
Pathagenic bacteria—nonhost plant	+	—
Saprophytic bacteria—plant (all)	—	—

It seems that HR is the most important defence reaction of plants which exists in all incompatible host—pathogen relations.

Bacteria seem to have an advantage over fungi and viruses in the study of the HR of plants. The most important reasons are:

a) Bacteria can be grown easily and rapidly on simple culture media.

b) They can easily be counted. Densitometry is a rapid means of adjusting the cell number of the inoculum.

c) The injection-infiltration method makes it possible to introduce a definite number of bacteria into intact plants.

d) Population changes during the reaction in the plant can be followed by the agar-plate count technique.

e) With concentrated inocula, the bacteria infiltrated into the intercellular spaces will spread uniformly in the tissues. In this way, all plant cells come into contact with the pathogen simultaneously and, hence, the HR starts in all affected cells approximately at the same time.

f) By injecting antibiotics into the intercellular spaces, multiplication can be stopped or bacterial cells can be killed at will.

If we could know the mechanism of the HR, we would obtain not only some informations on biochemical and physiological mechanisms of this defence reaction, however, we would be able to understand the basic concepts of pathogenesis as well as resistance.

In our investigations we used tobacco plants as host and *Pseudomonas syringae* (pathogenic to stone fruit trees) and *Ps. phaseolicola* (pathogen to bean) as incompatible pathogens to tobacco. In these systems, the HR developed by 8 – 10 hr after infiltration of bacteria into the plant intercellular spaces. It was possible to distinguish three phases during the development of the HR as follows: induction time, latent period and the collapse of host cells.

Induction Time

Induction time is that period which is necessary for the bacterium to initiate the HR. It is possible to measure the length of the induction time infiltrating antibiotics into the intercellular spaces in different intervals after inoculation. In our experiments, which were made with *Ps. phaseolicola* and tobacco leaf the length of induction time is 3 – 4 hours. This depends on the physiological condition of the bacterium but it is independent of the host plant. If the bacteria are killed in the plant tissue during the induction time no HR develops.

The concentration of inoculum does not influence the time of the appearance of the HR.

Our investigations show that multiplication of the bacterium during the induction time is not an important factor to induce HR, and that the controlling factors once activated in the host are irrevocable.

It would seem that the hypersensitive response can be induced only by living bacteria, though STALL and COOK (1968) and SEQUEIRA and AINSLIE (1969) prepared bacterial cell-free extracts, which were able to produce necrosis. However, with this cell-free extract it is very difficult to explain the host specificity of bacteria. Unfortunately, we have no more details about this very interesting experiments.

Latent Period

The latent period takes time from starting of plant reaction to tissue collapse. This period is independent of the presence of the living bacterium. Namely if we kill the bacterial cells by antibiotics during the latent period, the HR will appear. Therefore, if the controlling factors are once activated in the host tissue at the end of the induction time, the defence reaction continues with the presence of living bacterial cells.

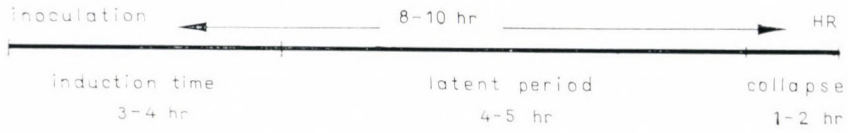

Fig. 1. Development of the hypersensitive reaction (HR)

During this period, the host is symptomless but some physiological and biochemical changes occur, for instance, the rate of respiration increases and it reaches the highest rate before tissue collapse appears (NÉMETH and KLEMENT, 1967).

The latent period is too short, therefore, there is not enough time for the development of the "slow" biochemical reactions. Most probably this high speed is the reason why an increase in polyphenoloxidase or peroxidase activity, the most widespread biochemical symptom of many other hypersensitive host-parasite complexes (fungi and virus), is not observed in the bacterial system (NÉMETH et al. 1969).

Tissue Collapse

After the latent period tissue collapse is rapid (1–2 hr) and dramatic and clearly suggests a loss of turgor in the affected cells, because the permeability of the host cell membrane is changed (GOODMAN, 1968). By measuring the change of permeability of host tissue electrolytically, one can see the rapid permeability increase at the end of the latent period. The highest point appears at the beginning of the period of the tissue collapse. If permeability of cell membranes changes, this way different ions come out from the cell plasma. One can suppose phenols also pass through the membranes of the vacuole. These phenol compounds may be oxidized in the cytoplasm causing cell death and finally tissue necrosis.

We are summarizing the development of the HR in Fig. 1.

We should like to point out that the observed physiological and biochemical changes in the host tissue during the development of the HR are not the initial causes, but the consequences of the HR. E.g. an increase in ammonia or ethylene

content, which was recently shown in the necrotized tissues (LOVREKOVICH, et al., 1970, 1971) is also a consequence of the HR.

The basic question is, how can the bacterium induce the HR in the incompatible host *only*. It seems that we can get the answer if we focus attention on the most important period of HR, namely, the induction time and the early part of the latent period.

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Effect of High Temperature and the Age of Bacteria on the Hypersensitive Reaction of Tobacco

By

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In the development of the hypersensitive reaction (HR) three phases can be distinguished: induction time, latent period and tissue collapse. The age of bacterium is an important factor in determining the length of induction time. The latent period remains unchanged. HR does not appear if the plant is kept at 37°C in the first 5 hrs after inoculation. The results show that, the early period of the latent phase is sensitive to high temperature (37°C).

In the previous paper (KLEMENT, 1970) the development of the hypersensitive reaction (HR) induced by plant pathogenic bacteria was discussed. In this paper three important phases were distinguished in the development of HR; induction time, latent period and cell collapse.

The basic questions, namely, how can bacteria induce HR in incompatible hosts only and what kind of physiological mechanisms are induced in the host by bacteria in the early part of the latent period, are unknown. This paper wants to give some information about the early phase of the development of HR.

Materials and Methods

To determine the period of the induction time *Nicotiana tabacum* L. was grown under ordinary greenhouse conditions until five to six expanded leaves developed. Middle, fully expanded leaves were used in most of the experiments. *Pseudomonas phaseolicola* isolated from halo-blight affected bean leaves were grown on nutrient broth agar. After 24 hr, the cells were suspended in sterile tap water and the suspensions were adjusted applying a densitometer to contain 10^8 cells/ml. The suspensions were infiltrated into half fully expanded tobacco leaves by means of a hypodermic syringe fitted with a fine needle (KLEMENT, 1963).

After injection the excess of water was evaporated from the tissue. To kill the bacteria in the leaf tissue at different intervals after the inoculation a solution of streptomycin at a concentration of 1000 μg/ml was injected into the treated (infected) leaf parts (KLEMENT and GOODMAN, 1967). Plants were similarly injected only with streptomycin solution or with water, served as controls. The treated leaves were incubated at 25°C and illuminated for 8 hrs daily.

The influence of physiological conditions of bacterial cultures on the length of induction time was investigated. Tobacco leaves were injected with suspensions of young and old cultures of *Ps. phaseolicola*. The bacterium was grown on nutrient broth agar 1,10 and 20 days. The bacterial cells were suspended in sterile tap water and the number of living cells were determined by application of the plate count method. The inoculum contained 10^8 cells/ml.

To investigate the effect of high temperature on HR, inoculated tobacco plants remained in the laboratory at 25°C and were put at 1 hr intervals in an incubator at 37°C.

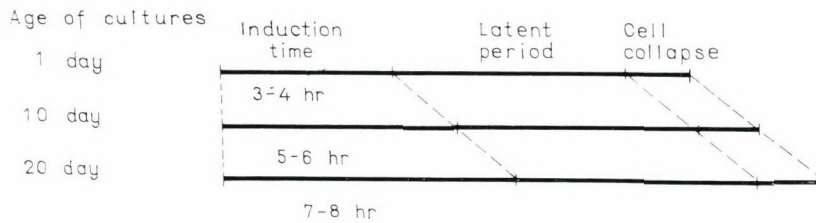

Fig. 1. Effect of the age of bacteria on the length of development of the HR

Results

It had been shown that only living, incompatible bacterial cells are able to induce HR in the plant (KLEMENT, FARKAS and LOVREKOVICH, 1964). *Ps. phaseolicola* pathogenic to bean but incompatible with tobacco induced HR in tobacco plant by 8–9 hr after infiltration. It was possible to measure the length of induction time infiltrating streptomycin into the intercellular spaces to kill the bacterium in different intervals after inoculation. When bacteria were killed during the first 3–4 hrs after inoculation, HR did not appear. However, when streptomycin was injected after 3–4 hrs HR appeared by 8–9 hr after infiltration of the bacterium. These experiments demonstrated that, in our system, the length of induction time was about 3–4 hrs. After this period, during the latent phase, living bacterial cells are not necessary for the development of HR.

We were able to show that the age of bacterial cultures also had an influence on the time of appearance of HR. Using 1, 10 or 20-day-old cultures, HR appeared 8–9 hrs, 10–11 hrs or 12–13 hrs after inoculation, respectively. HR was 3–4 hrs late when the bacterial culture was an old one.

We investigated the effect of age of bacteria in the first period (induction time) of the development of HR. These experiments were carried out by infiltrating streptomycin into the intercellulars at different intervals after bacterial inoculation with young or old cultures. As is seen in Fig. 1, only the induction time was influenced by the age of bacteria. The latent period and cell collapse did not change.

In a preliminary paper (KLEMENT and GOODMAN, 1967) we reported

that the HR was inhibited by high temperatures. When the inoculated tobacco plants were kept in an incubator at 37°C the HR did not appear. In this study we tried to find out which phase of the development of HR was inhibited by high temperature.

To determine the effect of high temperature on HR, tobacco plants were infiltrated with suspension of *Ps. phaseolicola* (10^8 cells/ml at 25°C low temperature) and put into an incubator at 37°C (high temperature) in 1 hr intervals during the 8-hr incubation period (Table 1).

Table 1

Appearance of the HR on infiltrated tobacco leaves incubated in various periods at 25°C and 37°C

Periods of low temperature at 25°C		Periods of high temperature at 37°C	HR
hr		hr	
Induction time	0	8	−
	1	7	−
	2	6	−
	3	5	−
	4	4	−
Latent period	5	3	+
	6	2	+
	7	1	+
	8	0	+

HR did not develop when plants were kept in an incubator at 37°C for at least 5 hrs after inoculation. After this interval high temperature had no effect on the appearence of HR.

In another experiment bacteria were killed by streptomycin in tobacco leaves 4 hrs after inoculation, i.e. 1 hr after the induction time. After this treatment plants were put in 37°C. In this case HR also did not develop, demonstrating that high temperature (37°C) has no effect on the bacteria or on the induction of HR. One can conclude from this experiment that the thermo-sensitive period exists in the early part of the latent period.

Discussion

These experiments confirmed our previous statement according to which it is possible to differentiate between three phases (induction time, latent period and cell collapse) during the development of HR.

Induction time is a period necessary for the bacterium to initiate HR. It would seem that this period is independent from the host, but it depends on the

metabolic activity of bacterial cells. E.g. young culture induces HR in the first 3–4 hrs but this period is twice as long when we apply old cultures. Because the generation time of *Ps. phaseolicola* is about 4–5 hrs in vitro, it seemed that the multiplication of the bacterium might be an important factor in the induction of HR. However, it was impossible to detect any multiplication of *Ps. phaseolicola* in tobacco tissue during this period. The factor which induces HR is unknown. The only thing we know is that young cultures, being more active, metabolically induce HR earlier than the old ones.

If the HR-inducing factors are once activated in the host tissue at the end of the induction time or at the beginning of the latent period the defence reaction continues without living bacterial cells. In our experiments it was shown that only the first two hours of the latent period represented the sensitive time to high temperature (37°C). This temperature did not affect the factors of induction but did affect the biochemical and physiological processes of HR induced in the plant.

The inhibition of HR by 37°C was also observed, when plants were inoculated with viruses (Ross, 1961, Martin & Gallet, 1966).

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Über den Einfluß von Pektinmethylesterase bei der bakteriellen Infektion pflanzlichen Gewebes

(Vorläufige Ergebnisse)

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Infections of *Vicia faba* with different virulent bacteria obviously depended on the stage of development of the plants. Therefore we have examined a possible correlation with the pectinmethylesterase production of the leaf tissue. When the plants were in the 8-leaf stage a marked increase in PME-activity from the 1st to the 8th leaf could be observed; the resistance against injected bacteria showed the same tendency.

In den letzten Jahren ist das Problem der enzymatischen Zersetzung von Pflanzengewebe durch phytopathogene Mikroorganismen Gegenstand zahlreicher Untersuchungen und Überlegungen geworden. Zusammenfassende Arbeiten liegen u. a. vor von STARR (1959) und WOOD (1960), aufschlußreich ist die eingehende Diskussion von BATEMAN u. MILLAR (1966). Der Abbau von Pektinen erfolgt in erster Linie hydrolytisch unter sauren Bedingungen durch endo-Polygalacturonasen. Ein Beispiel für eliminative Spaltung durch endo-Pektinsäuretranseliminase wurde kürzlich von MOUNT et al. (1970) beschrieben. Pektinmethylesterase (PME) bewirkt die Demethylation der Methylester von Polygalacturonsäureketten. Ihr Einfluß auf den Abbau ist umstritten. Nach SMITH (1958) soll sie maßgeblich am Fäuleprozeß beteiligt sein. BATEMAN und MILLAR (1966) vertreten hingegen die Ansicht, daß PME-Aktivität dem Infektionsablauf entgegenwirkt, insofern als bei der Freisetzung der Carboxylgruppen eine Valenzbindung zwischen den Pektinketten durch im Saftstrom vorhandene Ca-Ionen erfolgt. Die Pektinketten rücken dadurch laufend stärker zusammen und verfestigen sich, während immer weniger Wasser in dem Netzwerk enthalten ist. Die hydrolytische Spaltung durch Polygalacturonasen erscheint schließlich kaum noch möglich. Für die Annahme spricht, daß in der Regel gefestigtes Blattgewebe älterer Pflanzen gegenüber einer Infektion widerstandsfähiger ist als junges, was bereits früheren Autoren aufgefallen war (s. STAPP 1958).

Wir haben unsererseits bei Infektionsversuchen mit verschiedenen Bakterien an Testpflanzen eine Abhängigkeit des Infektionsverlaufes von der Entwicklung der Pflanzen festgestellt und daraufhin an *Vicia faba* geprüft, ob ein Zusammenhang mit der Pektinmethylesterase-Aktivität der Blätter besteht.

Methoden

Nährlösung für Bakterien. a) KH_2PO_4 3.0 g; $CaCl_2 \times 2\ H_2O$ 1.0 g; $MgSO_4 \times 7\ H_2O$ 0.25 g; $(NH_4)_2SO_4$ 3.75 g; Na-Glutamat 3.75 g; aqua dest. 1000 ml; pH 6.8. b) Pektin aus Citrus $OCH_3 > 7\%$ bzw. 70%, aus Apfel OCH_3 65%, 10 g; aqua dest. 1000 ml; pH 6.8. a : b = 400 : 100 ml.

Schüttelkultur. 100/Min. bei 23–28 °C, 48–50^h. Die Bakterien wurden unter Kühlung abzentrifugiert, das Supernat im Vakuum bei 30 °C auf 1/10 Volumen eingeengt.

Blattextrakte. Abgeschnittene Blätter wurden bei −18 °C eingefroren, zur Aufarbeitung bei Zimmertemperatur aufgetaut und die Faserteile entfernt. Den gewonnenen Gewebebrei haben wir mit äquivalenter Menge 0.1 m Na_2HPO_4-Lösung über Nacht eluiert, dann die festen Bestandteile durch Filtration und Zentrifugation unter Kühlung abgetrennt.

Kulturfiltrate und Blatteluate wurden über Nacht in der Kälte gegen demineralisiertes Wasser dialysiert, bis zum Gebrauch tiefgefroren.

PME-Aktivität. Bestimmung erfolgte durch fortlaufende Titration mit 0.01 *N* NaOH auf pH 7.0 bei 30° C. Reaktionsgemisch: Pektin 1%, NaCl 0.1 m; Bromthymolblau 0.04% in Äthanol 20%; Kulturfiltrat bzw. Eluat (9 : 1 : 0.1 ml).

Relative Aktivität. Während 2 h Inkubation freigesetzte mg Carboxyl je ml Kulturfiltrat bzw. Eluat multipliziert mit 100 (rel. Akt. = $0.01 \times 45 \times X \times 10$; X = ml 0.01 *N* NaOH).

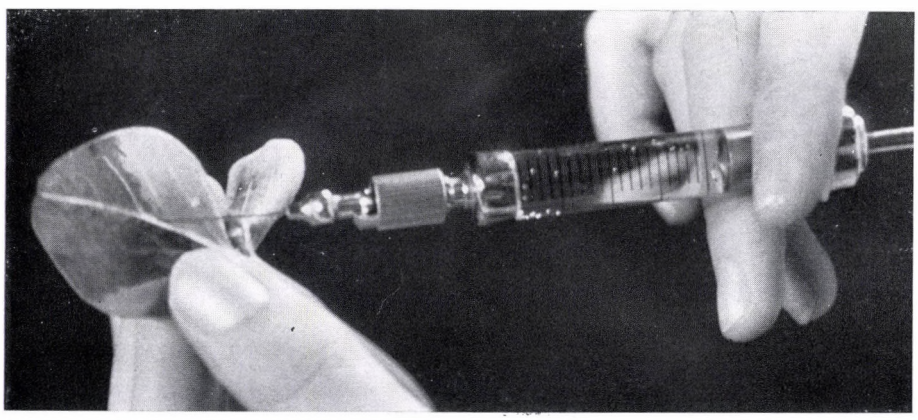

Abb. 1. Injektion einer Bakterien-Suspension in die Interzellularen eines Fiederblattes an *Vicia faba*

Infektion. Vicia faba wurde im Gewächshaus bei 20–26 °C und 60–80% rel. Feuchte herangezogen und die Blätter infiziert, indem nach der Methode von KLEMENT (1963) vermittels einer Injektionsspritze 0.1–0.2 ml Bakteriensuspension durch die Hauptader in die Interzellularen eingebracht wurden (Abb. 1). *Vicia*

faba besitzt wechselständige, einfach gefiederte Blätter; jeweils eines der Fiederblättchen wurde infiziert. Die Dichte der Bakteriensuspension erwies sich als wesentlicher Faktor. Bei zu hohen Zellzahlen lief die Reaktion des Blattgewebes zu schnell, bei zu kleinen Mengen zu langsam ab, optimal waren $10^4 - 10^5$ Zellen/ml. Die Pflanzen wurden zweimal täglich bonitiert.

Ergebnisse

PRUNIER und KAISER (1964) sowie LANGE und KNÖSEL[1] haben eine Vielzahl phytopathogener Bakterien aus verschiedenen Gattungen auf Fähigkeit zur Pektinmethylesterase-Produktion getestet mit weitgehend negativem Ergebnis. Nur wenige Organismen wiesen eine nennenswerte PME-Aktivität auf; einen Einfluß auf die Virulenz halten LANGE und KNÖSEL für unwahrscheinlich.

Tabelle 1

Pektinmethylesterase-Aktivität von Kulturfiltraten verschiedener phytopathogener Bakterien sowie von Blattauszügen einiger Testpflanzen (Bestimmung an Reaktionslösungen mit unterschiedlich veresterten Pektaten)

Bakterienspezies bzw. Pflanzenspezies	Rel. PME-Aktivität. Grad der Verestterung		
	7% (Citrus)	70% (Citrus)	65% (Apfel)
Ps. solanacearum NCPPB 1029	21	18	28
Ps. solanacearum NCPPB 1400	68	77	57
Ps. phaseolicola ATCC 11365	—	—	—
Ps. phaseolica ATCC 11355	—	—	—
Ps. syringae Ho	—	—	—
Ps. lachrymans Ho	—	—	—
X. begoniae Ho	9	4	9
X. vesicatoria NCPPB 1616	—	—	—
Phaseolus vulgaris	162	157	140
Vicia faba	239	338	176
Physalis floridana	36	54	32

Wir haben nunmehr einige virulente Stämme und geeignete Testpflanzen auf PME-Produktion geprüft, wobei wir zur Induktion und Reaktion unterschiedlich veresterte Pektine aus Citrus und Apfel verwandten. Das Ergebnis zeigt Tab. 1. Mit Ausnahme von *Ps. solanacearum* wiesen die Kulturfiltrate keine Pek-

[1] im Druck.

tinmethylesterase bzw. nur sehr geringe Aktivität auf. Herkunft und Veresterungsgrad des Pektins übten offenbar darauf keinen Einfluß aus. Die Aktivität der Blattelutate ist hingegen als hoch zu bezeichnen, insbesondere bei *Vicia faba.* Die Reaktion wurde von der Art des Pektins insofern beeinflußt, als Apfelpektin jeweils die niedrigsten Werte lieferte.

Wir haben vergleichende Infektionen und Aktivitätsbestimmungen an Blättern verschiedenen Alters vorerst nur an *Vicia faba* durchgeführt, da sich diese Pflanze auf Grund ihres Habitus am besten dafür eignet. Unter den beschriebenen Anzuchtbedingungen setzt die Blüte etwa nach Entfaltung des 8. – 10. Blattes ein. Im 8-Blattstadium wurden die Blätter in der beschriebenen Weise infiziert und parallel auf PME-Aktivität untersucht. Das Blattgewebe reagierte auf die Injektion in der Regel mit deutlichen Symptomen. Erstes Zeichen war eine Aufhellung der Spreite, dann erfolgte eine leicht gelbliche Verfärbung und das Gewebe begann zu nekrotisieren, meistens vom Blattrand ausgehend. Mit schwärzlicher Verfärbung schritt die Nekrose fort, bis das gesamte Fieblättchen abgestorben war und abgestoßen wurde (Abb. 2). Als manifestiert betrachtet haben wir die Infektion, wenn das Eintreten einer Nekrose unverkennbar war. Dieser Zeitpunkt nach der Injektion wurde vermerkt.

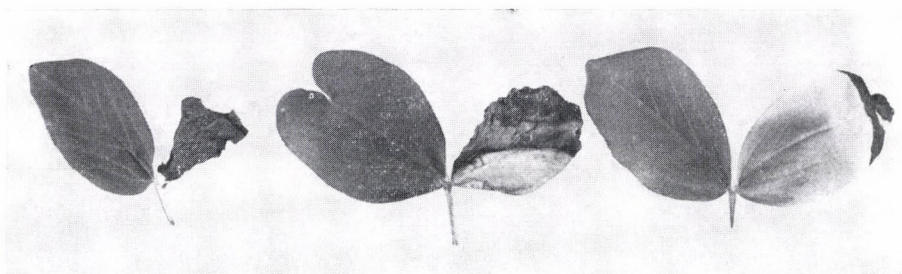

Abb. 2. Verfärbung und Nekrose bei Fiederblättern von *Vicia faba* nach Injektion von *Ps. lachrymans*

Unter Hinweis auf Abb. 3 wäre festzustellen, daß im 8-Blattstadium die PME-Aktivität des 1. und 2. Blattes relativ schwach war, vom 3. Blatt ab stieg sie merklich an und erreichte beachtliche Werte. Wir haben dazu vergleichend die Reaktion der Blätter auf Injekton mit 6 virulenten Bakterienstämmen verfolgt. Eine Abhängigkeit im zeitlichen Erscheinen der Symptome von der Blattfolge war unverkennbar, dabei bestanden zwischen den Organismen gewisse Unterschiede. Das 1. und 2. Blatt waren gegenüber der bakteriellen Infektion wenig oder gar nicht widerstandsfähig, was sich in schneller Nekrotisierung zeigte. Die folgenden Blätter waren widerstandsfähiger, da sich der Zeitpunkt der Nekrotisierung mehr oder weniger verzögerte. Am deutlichsten war dies bei *Ps. lachrymans* der Fall; das 1 Blatt reagierte bereits am 2. Tag, die folgenden Blätter merklich später und vom 6.

Blatt ab traten keine Nekrosen mehr auf. Beide Stämme von *Ps. phaseolicola* sowie *X. begoniae* zeigten gleichfalls die Tendenz der Verzögerung des Infektionsverlaufes mit zunehmender Blattfolge. Der benutzte Stamm von *Ps. syringae* bewirkte eine sehr starke und schnelle Reaktion des Blattgewebes; nach 1½ Tagen waren sämtliche injizierte Blätter nekrotisch. Bei *X. vesicatoria* war hingegen die Tendenz möglicherweise gegenläufig, was keineswegs übersehen werden soll.

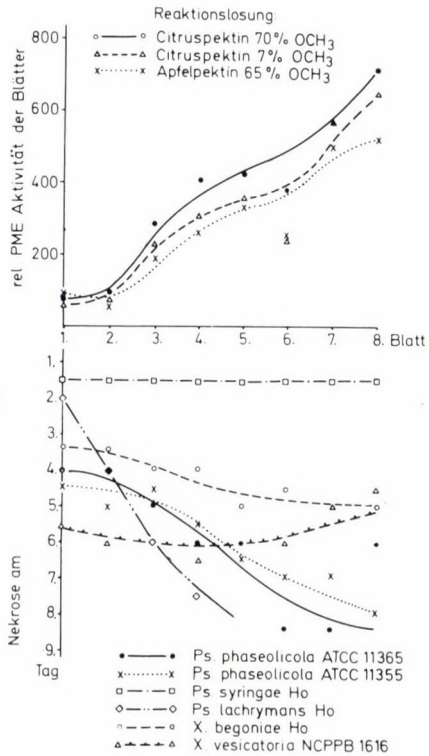

Abb. 3. Pektinmethylesterase-Aktivität der Blätter von *Vicia faba* im 8-Blattstadium und Infektionsverlauf nach Injektion von Bakterien-Suspension

Wir nehmen gewiß nicht an, daß der hochgradig komplizierte Vorgang der Infektion allein durch ein Enzym entscheidend beeinflußt werden kann. Die mitgeteilten Befunde lassen jedoch eine gewisse Korrelation zwischen der PME-Aktivität des Blattgewebes und der Widerstandsfähigkeit bei *Vicia faba* erkennen. Unbedingt vergleichbare Feststellungen machten BATEMAN u. LUMSDEN (1965) an Hypocotylen von *Phaseolus vulgaris*, deren Gewebe mit zunehmender Reifung gegen *Rhizoctonia solani* resistenter wurde, was die Autoren auf die Überführung von Pektin im Calziumpektat zurückführten; dieses setzt entsprechende PME-Aktivität voraus. Nach den Untersuchungen von KLEMENT u. LOVREKOVICH (1961)

wissen wir, daß Bakterien im Wirtsgewebe antiinfektionelle Reaktionen hervorrufen. Bei *Nicotiana tabacum* wiesen NÉMETH et. al. (1969) nach Injektion von *Ps. syringae* Veränderungen in der Produktion mehrerer Enzyme nach. Wir prüfen zur Zeit, ob durch das Einbringen von Bakterien in das Blattgewebe von *Vicia faba* auch die PME-Aktivität gesteigert wird. Wenn dies der Fall ist, so wäre eine enzymatische Abwehrreaktion gegeben.

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Ecology of Insect Pests

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Zur Kenntnis bestimmender Faktoren im Ökosystem als Voraussetzung für den sinnvollen Einsatz von Bekämpfungsverfahren gegen blattfressende Obstbaumschädlinge

Von

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The regular chemical plant protection system used in intensively treated orchards, leads to 10—15 sprayings in course of a vegetation period. The extensive use of chemicals promotes the development of resistance and usually destroys the majority of regulative biotic factors. There is an ever stronger need to carry out the plant protection measures by saving the natural enemies of pests and enabling them to decimate the pests at low individual densities. This could be achieved in case of lepidopterous pests of fruit trees, whose larvae feed on the foliage, by using selective pesticides at optimal dates, with special reference to bacterial preparations. The optimal time for the use of the latter would be in the G.D.R. end of April begin of May. A rational combination of pesticides and plant protection measures has to be achieved for controlling the pest populations but also taking into consideration every other elements of the communities too. It would be outmost desirable to decrease the use of various pesticides in order to prevent pest resistance, residual problems and to increase the stability of ecosystems of the orchards.

Es werden gegenwärtig in der angewandten Entomologie und im Pflanzenschutz in zunehmendem Maße nicht nur die Einzelarten untersucht, sondern man studiert mehr und mehr die ursächlichen Beziehungen in der Biozönose, d. h., man schenkt dem Schädling im Ökosystem – im Komplex der Lebewesen mit den Kulturpflanzen und den übrigen Umweltfaktoren – stärkere Beachtung. Zur Erfassung solcher sehr vielschichtigen, in Ort und Zeit wechselnden Komponenten, die in einem ständig pulsierenden System wirksam sind, bedarf es sehr tiefgründiger Untersuchungen, die meistens auch nach langjährigen Arbeiten nur einen Stein zu einem größeren Gebäude beitragen können.

Die Populationsdichte wirtschaftlich wichtiger blattfressender Obstbaumschädlinge, wie z. B. Euproctis chrysorrhoea L., Operophtera brumata L., Lymantria dispar L., Malacosoma neustria L., Yponomeuta malinella ZELL., wird von den sich zum Teil gegenseitig bedingenden abiotischen, biotischen und anthropogenen Faktoren entscheidend beeinflußt. Die Schädlingsdichte wird unter den klimatischen Bedingungen Mitteleuropas sehr wesentlich durch die Wirkung von Parasiten und Krankheitserregern im Verlaufe der Raupenentwicklung dezimiert (Abb. 1).

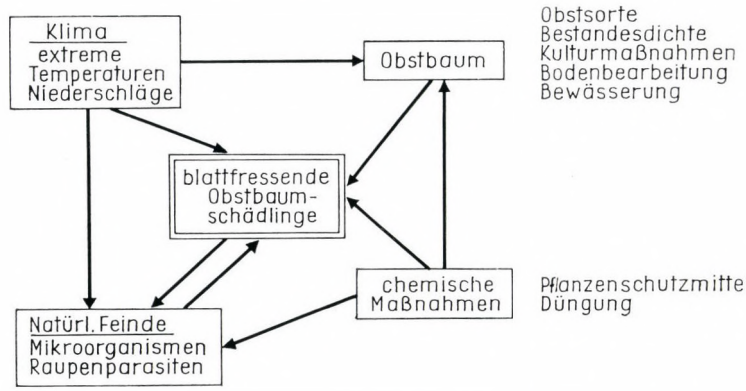

Abb. 1. Wichtige Faktoren, die das Vorkommen blattfressender Obstbaumschädlinge beeinflussen

Einige dieser blattfressenden Schädlinge zeigen die Tendenz, daß nach zweijähriger Kulminationsphase, vor allem durch die Tätigkeit natürlicher Feinde die Massenvermehrung zusammenbricht.

Außer abiotischen Einflüssen, die direkt oder indirekt auf den Schädling einwirken, kommt der Wirtspflanze in ihrer Stellung im Pflanzverband oder -bestand und den die Wirtspflanze beeinflussenden Faktoren eine entscheidende Rolle für eine Schädlingsvermehrung oder -begrenzung zu. Im Blickwinkel der »trophischen« Theorie von RUDNEV (1958) haben die für das Pflanzenwachstum optimalen Bedingungen eine entscheidende Bedeutung. Durch ungenügende Versorgung der Pflanzen mit Nährstoffen und Wasser, ebenso durch gewisse anthropogene Faktoren, wie Rauche und Einwirkung anderer chemischer Substanzen, sind Veränderungen des physiologischen Zustandes der Pflanze bekannt. Zu wenig wissen wir aber über eine möglicherweise damit verbundene physiologische Veränderung der Schädlingspopulationen hinsichtlich Fruchtbarkeit, wechselnden Erbkonstitutionen u. a.

Die Kenntnis der *Wirksamkeit natürlicher Feinde* in den zu behandelnden Obstbauanlagen ist eine wesentliche Voraussetzung, um integrierte Bekämpfungsverfahren sinnvoll einzusetzen. Die Höhe der Sterblichkeit, die durch Entomophagen verursacht wird, hängt sehr wesentlich davon ab, zu welchem Zeitpunkt das Material im Freiland eingesammelt wird, in welchem Gebiet und in welcher Anlage die blattfressenden Schädlinge und entsprechenden Nützlinge untersucht werden. Um aber zu verallgemeinerungswürdigen Aussagen zu kommen, ist es notwendig, von den früher allgemein üblichen Parasitenlisten abzukommen (FANKHÄNEL, 1957; 1961). Es ist erforderlich, gründlicher die Effektivität der Nützlingsarten zu kennen, den Zeitpunkt des Vorkommens der Hauptparasitenarten zu ermitteln, um daraus Schlußfolgerungen für den richtigen Einsatz nützlingsschonender Bekämpfungsmittel und -verfahren abzuleiten (Abb. 2). Aus den

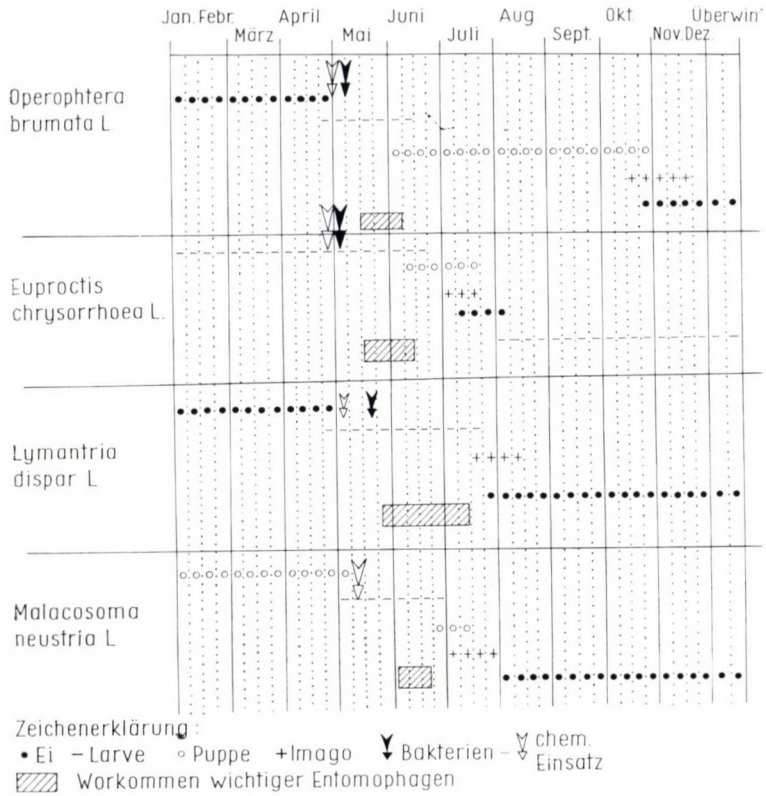

Abb. 2. Phänogramm wichtiger blattfressender Obstbaumschädlinge

erhaltenen Phänozyklen besonders effektiver Entomophagenarten in Verbindung mit der Entwicklung wirtschaftlich wichtiger blattfressender Obstbaumschädlinge ist ersichtlich, daß die wirksamsten Parasitenarten Ende Mai und im Juni als Imagines anzutreffen sind. Im Hinblick auf eine Schonung und Förderung endemischer Nützlinge unter Beachtung der im Ökosystem wirkenden Faktoren ist es für das Gebiet der DDR empfehlenswert, chemische Bekämpfungsmaßnahmen mit möglichst selektiv wirkenden Mitteln gegen diesen Komplex blattfressender Obstbaumschädlinge je nach Witterungsverhältnissen Ende April bis Anfang Mai einzusetzen. Selbstverständlich darf man diesen Schädlingskomplex nicht losgelöst betrachten vom Auftreten und der Bekämpfung anderer frucht- bzw. triebschädigender Organismen in der Obstanlage. Aber die umfassende Kenntnis des Schädlings–Nützlingskomplexes, in den von TANSLEY (1935) dargelegten Grundsätzen zur Ermittlung des Ökosystems schafft entsprechende Voraussetzungen, um

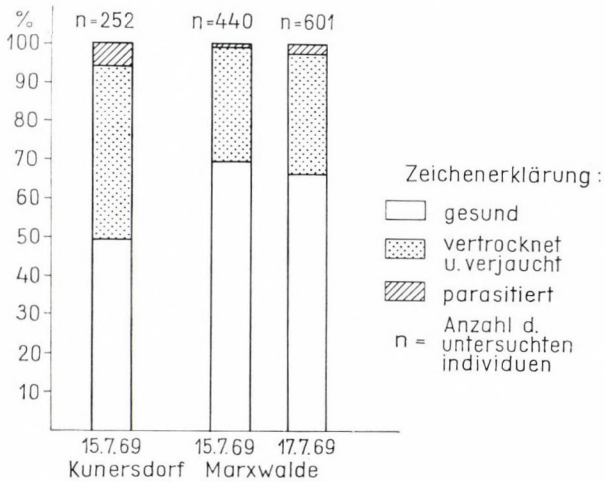

Abb. 3. Der Gesundheitszustand des Schwammspinners *Lymantria dispar* L. in Kunersdorf und Marxwalde (Krs. Seelow) im Jahre 1969

den Einsatz von Pflanzenschutzmitteln und -verfahren in Zeit und Ort so durchzuführen, daß die wichtigen Elemente der natürlichen Feinde erhalten bleiben, die in der Lage sind, Restbestände von Schädlingen reduzieren zu können und auch in den folgenden Generationen regulativ einzugreifen.

Bei einigen blattfressenden Obstbaumschädlingen sind einzelne Parasitenarten durchaus als Weiserarten zu benutzen, um über die beginnende Retrogradation der Schädlingsvermehrung Auskunft zu erhalten. So kann z. B. der Parasitierungswert der Schlupfwespe *Eupteromalus hemipterus* THOMS. als Anzeiger für den Zusammenbruch der Schädlingsvermehrung von Euproctis chrysorrhoea dienen. Die entsprechenden ökologischen, epidemiologischen Untersuchungen helfen sehr wesentlich, unnötig werdende Bekämpfungseinsätze rechtzeitig einzustellen.

Für die Prognose des zu erwartenden Schädlingsauftretens und für evtl. einzuleitende Bekämpfungsmaßnahmen ist es sehr wichtig, von Hauptschadgebiet zu Hauptschadgebiet den Gesundheitszustand und den Parasitierungsanteil einer Schädlingspopulation exakt zu ermitteln (Abb. 3). Am Beispiel von zwei Populationen des Schwammspinners ist gezeigt, daß die Einschätzung des Gesundheitszustandes Aussagen über den Anteil kranker bzw. parasitierter und vor allem gesunder Tiere gestattet. Selbstverständlich ist es gewöhnlich schwierig, eine allumfassende, verallgemeinernde und in ihren ursächlichen Beziehungen fundierte Beurteilung des insgesamt wirksamen Faktorensystems zu geben. Aber die Angaben über den Gesundheitszustand repräsentieren sich in seiner Wirkung letzten Endes in jenem Teil der Population, der aus gesunden Tieren besteht, die ihre Entwicklung über das Ei-, Raupen- und Puppen- bis hin zum Imaginalstadium abschließen.

Die derzeit in den Obstanlagen routinemäßig betriebenen Schädlingsbekämpfungen mit fast ausschließlich chemischen Mitteln, die in Intensivanlagen 10–15 Spritzungen pro Jahr erreichen, fördern sehr wesentlich eine schnellere Entwicklung resistenter Populationen, schalten gewöhnlich den größten Teil der regulativ wirkenden Elemente völlig aus, erhöhen den Anteil der Rückstände an Pflanzenschutzmitteln und verringern die Stabilität in Obstbaubiozönosen. Wir stimmen mit Schlußfolgerungen von PICKETT (1958) und STEINER (1968) überein, daß sich der Pflanzenschutz der Zukunft vor der Überbetonung chemischer Maßnahmen, besonders in der tierischen Schädlingsbekämpfung, freimachen und zu einem komplexen System von integrierten Bekämpfungsverfahren übergehen muß. Es kommt darauf an, durch gezielten Einsatz der Mittel unter Einbeziehung aller brauchbaren neuen Bekämpfungs- und Begrenzungsverfahren, seien es Methoden der biologischen, insbesondere der mikrobiologischen Bekämpfung, Verfahren der Sterilisation oder des Einsatzes von Juvenilhormonen, bei geringster Schädigung der biotischen Begrenzungsfaktoren einen optimalen Bekämpfungserfolg gegen die Schädlinge zu erreichen.

Die in den früheren Jahren gegen Forstschädlinge (FANKHÄNEL, 1962) und in den Jahren 1969 und 1970 gegen einige blattfressende Obstbaumschädlinge durchgeführten Einsätze mit Bakterienpräparaten (NGYUEN, V. D.) bestätigten die Vorteile in der Anwendung mikrobiologischer Präparate.

Insbesondere konnte auf die Schonung der Nützlingsfauna aufmerksam gemacht werden. Entscheidend für den wirkungsvollen Einsatz von Bakterien- und auch chemischen Mitteln ist die Wahl des richtigen Bekämpfungstermins (Abb. 2). Bei den blattfressenden Obstbaumschädlingen kann man durch einen einmaligen Einsatz von Bakterienmitteln und auch chemischer Präparate die Schädlinge wirksam treffen und dabei eine nützlingsschonende Schädlingsbekämpfung erreichen. Voraussetzung für eine hohe Effektivität der Bekämpfungseinsätze ist auf alle Fälle die gründliche Kenntnis der bestimmenden Faktoren im Ökosystem und deren Beachtung in den anzustrebenden integrierten Bekämpfungsverfahren.

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Frequency-dependency and the Maintenance of Polymorphism in Insect Populations

By

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Mating success was studied between strains of different geographical origins or between different mutations in several species of Drosophila. It appeared to be a function of the ratios of the two competing strains, with an advantage held by the rare type: in most cases, the males that are at a relative disadvantage when their frequency in the population is 0.50 or over, are at an advantage in mating when their frequency is only 0.20. The same phenomenon was found for larval selection in Drosophila melanogaster and Tribolium castaneum: when two types of eggs are in competition on a small amount of food, the ratio that hatches and gives adults is frequency-dependent, with an advantage held by the rare type. The consequence is that an equilibrium may happen for the frequency corresponding to the neutral selective value. When the majority form is at a relative disadvantage, its frequency decreases, but by becoming rare enough it has an advantage again and its frequency increases. The equilibrium is maintained by a series of fluctuations.

Any natural population shows a great deal of genetic heterogeneity that promotes adaptation when the environment changes. This makes the struggle against insects all the more difficult. Many genes, though disadvantageous when homozygous, are maintained in the populations in spite of natural selection. Neither mutation frequency, nor heterosis are sufficient to explain their maintenance. Another mechanism has been demonstrated: the advantage of the rare type in sexual competition and larval selection.

I. Sexual selection

Mating success has been studied between strains of different geographical origins or between different mutations, in several species of Drosophila.

Technique

The males and females of the two strains are kept together; then the females are placed in individual vials and the type of male they mated with is determined by examination of their progeny.

Another technique may be used: 12 pairs of each of the two strains in competition are put into a flat glass box; the copulations are recorded as soon as they occur.

A *coefficient of mating success* is defined as the ratio of the probabilities of a female to be inseminated by one or the other kind of males. Let *a* be the number of males of type 1 in the population, *b* the number of males of type 2, A and B the number of females inseminated by them; then the coefficient of mating success is:

$$K = \frac{A/a}{A/b}.$$

With random mating, K equals unity; K lower than 1 means that the males of type 1 are at a disadvantage; and K higher than 1, that the males of type 1 are at an advantage in mating.

Results

With the *Bar* mutation of *Drosophila melanogaster* (PETIT, 1951), K is frequency-dependent, but always at a disadvantage (Fig. 1). The *white* mutation gives more interesting results, as K is over one when *white* is rare (PETIT, 1958), (Fig. 2). It may be seen that the relative selective value is frequency-dependent and may be responsible for the maintenance of polymorphism: when *white* frequency is over 40%, *white* is at a disadvantage, so the frequency falls; but when it is under 40%, *white* is at an advantage, and its frequency rises; an equilibrium is maintained at 40% of *white* males.

SPIESS found the same kind of result with *D. persimilis* (1964, quoted by EHRMAN, 1966); so did EHRMAN et al. in *D. pseudoobscura* (1965–1966). EHRMAN and PETIT (1968) studied the advantage of the rare type in *D. willistoni*, *D. equinoxialis* and *D. tropicalis* (Table 1), and BORISOV has just discovered it in *D. funebris* (1970).

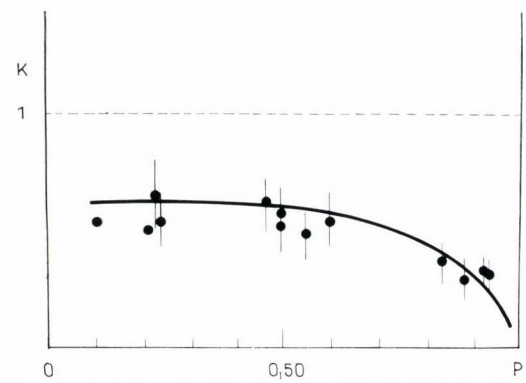

Fig. 1. The coefficient of mating success between *Bar* and its *wild* allele (abscissa: percentage of *Bar* males, ordinate: coefficient of mating success)

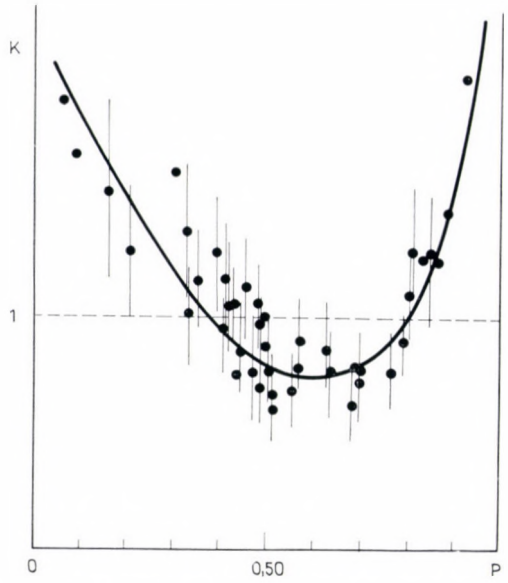

Fig. 2. The coefficient of mating success between *white* and *wild* allele

Table 1

The advantage of the rare type in the willistoni group of species (A B C D E are strains of different geographical origins)

Species	Ratio %	Coefficient of mating success		
		A×B	C×D	D×E
D. willistoni	20	1.1±0.3	2.4±0.4	4.2±0.5
	50	0.5±0.1	1.4±0.2	1.3±0.3
	80	0.5±0.2	0.7±0.2	0.7±0.2
D. equinoxialis	20	1.1±0.3	3.7± 0.4	1.4±0.3
	50	0.8±0.2	1.3±0.2	0.9±0.2
	80	0.2±0.1	0.7±0.2	0.5±0.2
D. tropicalis	20	2.0±0.4	3.6±0.5	2.2±0.4
	50	1.4±0.3	1.3±0.3	0.8±0.3
	80	0.7±0.2	0.6±0.2	0.5±0.2

II. Larval competition

A frequency-dependent selection has been discovered for larval competition in *D. melanogaster* (PETIT, 1966; KOJIMA and YARBROUGH, 1967; PETIT and ANXOLABEHERE, 1968; ANXOLABEHERE, 1971), and by SOKAL and KARTEN (1964) in *Tribolium castaneum*.

A *coefficient of viability* is defined as the ratio of the probability P of hatched imagos, divided by the probability p of the eggs of the same type : P/p. Another coefficient may be used that shows the interaction between the two ge-

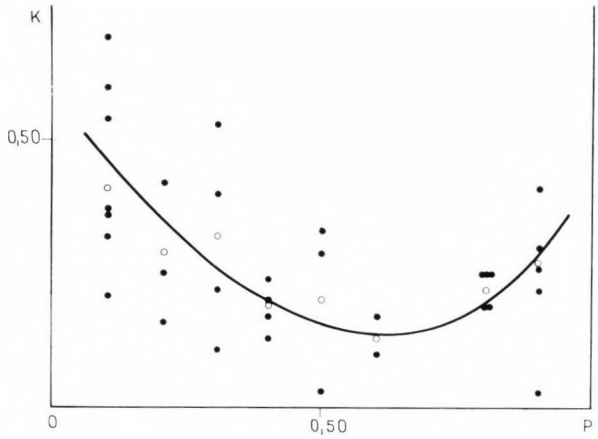

Fig. 3. The selective value of *white* versus *wild* in larval competition (abscissa: percentage of *white* eggs, ordinate: selective value)

notypes and gives the *selective value* τ of one genotype compared to the other; let P/p be the *coefficient of viability* of the genotype 1, Q/q, the *coefficient of viability* of the genotype 2, then the selective value τ is P/p : Q/q. The competition between *white* and wild has been studied (Fig. 3). It may be seen that the coefficients of viability are frequency-dependent. Most interesting results are given by the competition between *ebony* and wild (ANXOLABEHERE 1971) (Fig. 4). The selective value is greater than unity when the frequency of *ebony* in the population is under 15% and smaller when their frequency is over 15%. If larval selection were the only component of fitness, the equilibrium would be attained when the population contains 15% of *ebony* and 85% of wild, and maintained by a series of fluctuations around this value.

Other experiments carried out all prove larval selection to be frequency-dependent.

It seems that the advantage of the rare type may be applied to equilibrium situations between predators and preys, parasites and hosts and explain Volterra's equations. But it is still a model, and if frequency-dependency seems to be a general phenomenon responsible for the maintenance of genetic polymorphism in populations, it has to be tested in many different species and in natural populations. But that is the task of ecologists.

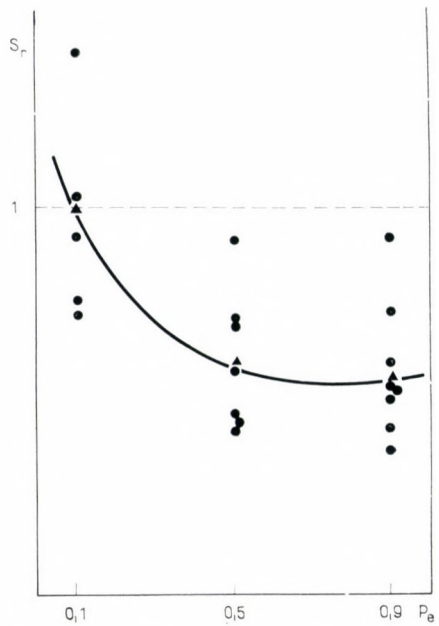

Fig. 4. The selective value of *ebony* versus *wild* in larval competition

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The Role of Natural Enemies of Fall Webworm (*Hyphantria cunea* Drury) in North America (Arkansas) and in Europe (Yugoslavia)

By

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The mass occurrence and damage of the fall webworm in 1967—69 in Europe has drawn again the attention of entomologists to this pest. In Yugoslavia at Subotica, Zemun and Bar (Adriatic Coast) the collection of 20 caterpillar nests was organized in 1967, 1968 and 1969 for each generation and locality, followed by a detailed laboratory analysis. In the U.S.A. (Northwest Arkansas) similar investigations were organized in 1965—66, from 3 localities. The insignificance of the autochtonous parasites in the reduction of the fall webworm population was most remarkable in Yugoslavia in all three localities. Contrary to this in North America the role of entomophagous insects was found very important in the reduction of the FWW population.

There have already been experiences about insect pests introduced before from one continent to the other, and about adaptation process of entomophagous insects on a new-introduced host. Thirty years on incidental introduction of the Fall Webworm (*Hyphantria cunea* Drury) from North America to Europe, parallel data have been collected on population dynamics of its entomophags.

These investigations were carried out in North America (3 localities in Arkansas State), and in Europe (3 localities in Yugoslavia) from 1965—1970. Natural enemies of the fall webworm caterpillars, and their number, were investigated on both continents. The study of other developmental stages of entomophagous insects has been in progress, Figs 1, 2.

Materials and Methods

Methods of caterpillar collecting in the field of Yugoslavia and North America differed to a certain degree. In Europe, only black race is present. In America, there are red and black races. Between them there are differences in their biological manifestations. However, entomophags are known to attack both races.

On a limited number of trees, in mentioned localities, collecting was organized of 20 caterpillar nests, where possible, for each generation. Then, detailed analysis of these nests was performed in the laboratory, at the end of each 3 days of collecting. In the moment of collecting, caterpillars were in their 3—4. instars. As such, they were fixed in Dubosque, and then dissected, or they were further

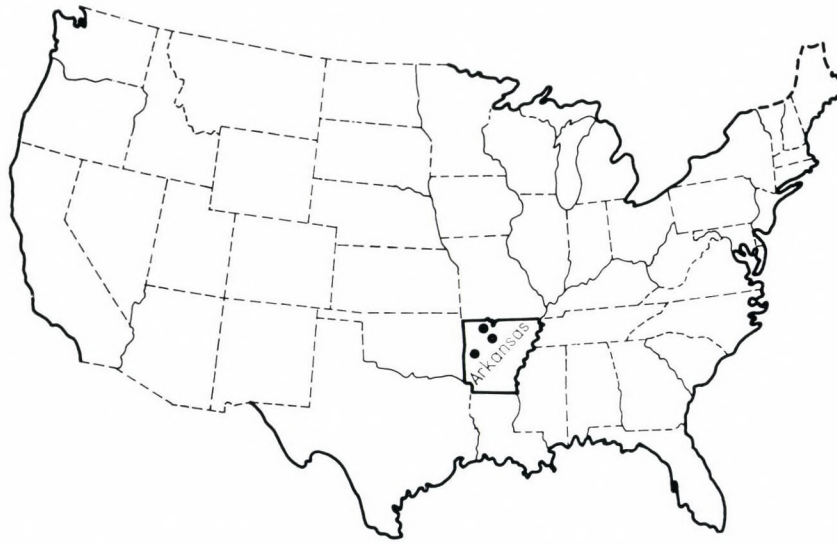

Fig. 1. Localities of collections in course of studies of natural enemies of Fall Webworm

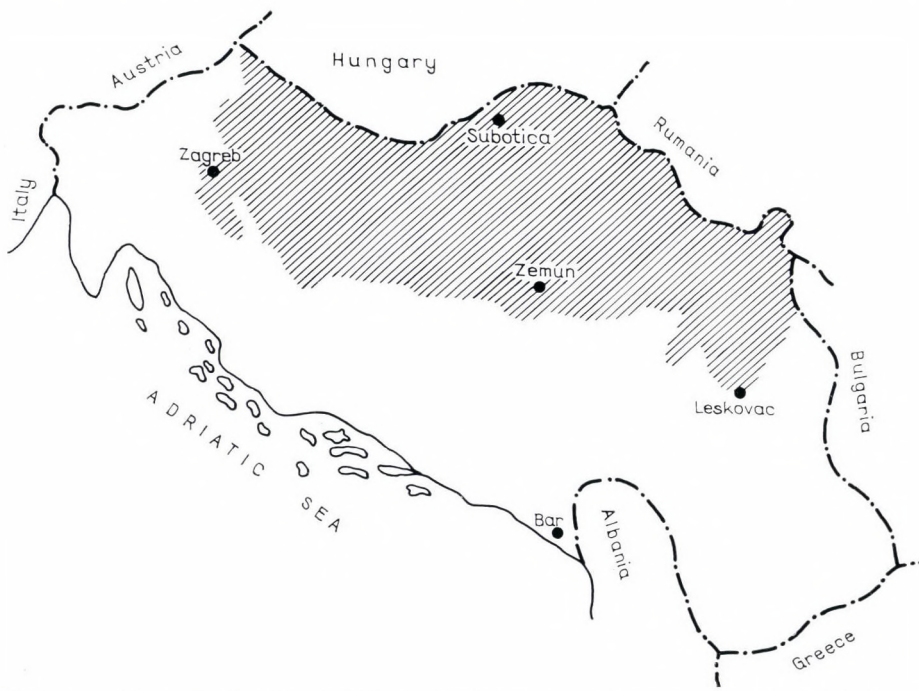

Fig. 2. Localities of collections in Yugoslavia, for studying the natural enemies of Fall Webworm

reared either to pupa, or to the emergence of primary, or secondary parasites, in order to find whether they were parasitized, or not. 37.583 caterpillars have been included in this work in North America, and 63.990 in Europe, not more than 111.573 for the last 5 years.

Localities in the USA were the following:

Huntsville, – typical urbanized territory

Pea Ridge – National Park – the battle field of the Civil War, today covered with forests and ornamental bushes.

White Rocky Mountain – Mountain – Forest Region.

Determination of reared parasites from Europe was made by OILB systematists, and of those from North America, by specialists of Washington Museum. It is my pleasant duty to express gratitude for their help.

Localities in Yugoslavia were the following:

Bar – Fresh focus in specific conditions of the South Adriatics, that is the first invasion of Fall Webworm into mediterranean climate region.

Zemun – In relation to the process of the fall webworm spreading, and in relation to climatic conditions, the mean rank was taken by this locality. Besides, it is urbanized.

Subotica – In the north of the country, and, in the same time, the first focus of the fall webworm in Yugoslavia on its spreading from Hungary, mainly on the terrain under forests and orchards.

Results

These investigations were aimed at the establishment of the number and efficacy of the fall webworm enemies in North America, as the country of this insect origin, and in Europe. Precise analysis and investigation of the material from 20 caterpillar nests permitted to compare data from each locality and continent. This way, besides comparing data by mathematic counts, the schematic presentation of research results in a model form was also permitted and realized.

On the base of basic tables with data stated for each caterpillar nest, and each year, synthetic tables were made.

Investigations of the species, number and efficacy of the fall webworm enemies on the two continents, as well as differences found, can be contributed by the following analysis, Table 1.

Conclusions

I am permitted by data from this analysis to find conspicuously more higher participation of the natural enemies of fall webworm in North America, than in Europe.

The fact that the presence of intermediate hosts as American species is con-

Table 1

Groups and numbers of the entomophags of *Hyphantria cunea* Drury larvae in North America and Europe

Continent	Groups of entomophags	Number of entomophags											
		1965 I	1965 II	1966 I	1966 II	total	1967 I	1967 II	1968 I	1968 II	1969 I	1969 II	total
North America	*Elachertus* sp.	47	—	131	40	218							
	Hyposoter sp.	595	350	111	120	1176							
	Meteorus sp.	18	9	39	41	107							
	Tachinidae	6	3	319	820	1148							
	Apanteles sp.	59	26	511	218	814							
	Podisus sp.	128	1	42	51	222							
	total	853	389	1153	1290	3685							
Europe	*Apanteles* sp.						—	8	—	—	—	—	8
	Tachinidae						13	84	64	2	—	—	163
	total						13	92	64	2	—	—	171

ditioned by American parasites, inspired us to undertake organizing the introduction of predators within the last three years. Egg predators were introduced, such as: *Coleomegilla maculata* (1968–6720 specimens), and *Podisus placidus*, *P. maculiventris* (1968–405 specimens), as predators of caterpillars. This was preceded by detailed laboratory investigations, and liberations in the open under cages.

I wish to point out that *C. maculata* overwintered in cages, in the surroundings of Zemun, in July 1970. In the following years, special attention will be paid to further following this species acclimatization in Yugoslavia.

For final conclusions it may be stated that:

1. There is, obviously, a much lower suppression role of autochtonal entomophags in reducing the populations of Fall Webworm in Europe than in North America;

2. A high participation of predators was observed among the natural enemies of Fall Webworm in North America, with nothing similar registered in Europe.

3. The introduction of North American parasites seems to be limited, as most of them are strictly associated to intermediary hosts not existing in Europe.

4. Study of the efficacy of European natural enemies, attacking other developmental stages of Fall Webworm is also in progress.

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Der Einfluß von Territorialverhaltensweisen von Mikrolepidopteren auf die Populationsdichte

Von

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In the course of studies carried out by using a bioacustic method, a particular territorial behaviour of larvae of *Sparganothis pilleriana* Schiff., *Pandemis ribeana* Hb. and *Chimabacche phryganella* Hb. could be observed. Obvious reference points seem to be given between this larval behaviour in a form of vibrational signals against intra- or interspecific competitors and the regulation of population density in a given area. This territorial behaviour was interpreted as an expression movement based on a conflict situation between escape- and attack appetence.

Larvale Territorialverhaltensweisen von Mikrolepidopteren wurden bisher nur vereinzelt näher untersucht (Russ, 1969).

Im Hinblick auf die mögliche Bedeutung solcher Verhaltensweisen auch im Rahmen moderner pflanzenschutzlicher Maßnahmen, ist es jedoch zweifellos von Vorteil, solche Untersuchungen mehr als bisher in Angriff zu nehmen. Im besonderen wäre dies auch aus folgenden Gründen besonders wünschenswert:

a) Mit einer stets zunehmenden Forschungstätigkeit auf dem Gebiete eines mehr ökologischen Pflanzenschutzes, bedarf es im besonderen einer starken Berücksichtigung des Studiums ökologischer Regulationsfaktoren und ihrer populationsdynamischen Wirkungsweise.

b) Im Hinblick auf eine fortschreitende Inangriffnahme von pflanzenschutzlichen Alternativmethoden zu den konventionellen chemischen Bekämpfungsverfahren wird in naher Zukunft in steigendem Maße auch einer genetischen Bekämpfung große Bedeutung zukommen. Die in diesem Zusammenhang erforderliche Massenzucht von Insekten auf kleinstem Raum bedarf zweifellos eines genauen Studiums ethologischer Eigenheiten solcher Schädlinge.

c) Darüber hinaus, besteht kein Zweifel, daß eine ganzheitliche Erforschung aller, die Populationsdichte steuernden Faktoren, d. h. also auch bestimmter Verhaltensweisen, allmählich auch zum besseren Verständnis von Massenvermehrungen führen kann.

Eigene Untersuchungen über das larvale Territorialverhalten von Mikrolepidopteren

Im Verlaufe von verschiedenen Untersuchungen über die Biologie des Springwurmwicklers, *Sparganothis pilleriana* Schiff. konnten an den Raupen Verhaltensweisen beobachtet werden, die auf das Vorhandensein eines ausgeprägten Territorialverhaltens hinweisen. Ähnliche Verhaltensweisen konnten auch noch an *Pandemis ribeana* Hb. und an *Chimabacche phryganella* Hb. beobachtet werden. Im folgenden soll über diese Untersuchungen berichtet werden.

Untersuchungsmethode

Die Untersuchungen wurden mit Hilfe einer einfachen bioakustischen Ausrüstung, bestehend aus einem Mikrophon, einem Tonbandgerät und einem Servoschreiber ausgeführt. Nachteilig wirkte sich bei dieser einfachen Beobachtungsmethode der Einfluß von Nebengeräuschen auf die Reinheit der Aufnahme aus. Trotz dieser Mängel konnte eine brauchbare Erkennbarkeit der sich als Geräusche darbietenden Verhaltensweisen erreicht werden.

Untersuchungsergebnisse

a) *Sparganothis pilleriana* Schiff. ist in allen europäischen Weinbaugebieten als Rebenschädling gut bekannt. Es ist auffallend, daß die Raupen dieser Art jeweils nur in Einzahl je Rebentrieb und dann meist nur an den Triebspitzen anzutreffen sind. Diese »solitäre« Lebensweise unterstreichen die Raupen vor allem dadurch, daß sie einfache Gespinste herstellen, in die sie grüne Pflanzenteile einbauen. Solche Gespinste werden von den Raupen nur sehr selten verlassen und meist nur dann, wenn ein neues Gewebe hergestellt werden soll. Die Gespinste dienen einerseits sicherlich als Schutz für die Raupen und andererseits als Freßterritorium. Wie Beobachtungen erkennen ließen, ist das Bestreben der Raupen, solche Gespinste herzustellen außerordentlich stark ausgebildet. Selbst nach längeren Hungerperioden wird, ehe Nahrung aufgenommen wird, mit der Herstellung des Gespinstes begonnen.

Wie nun unsere Beobachtungen zeigten, wird dieses Freßterritorium sowohl gegen intra- wie auch interspezifische Konkurrenten energisch verteidigt. Dieses Territorialverhalten manifestiert sich folgendermaßen:

Versucht z. B. eine arteigene Raupe, in ein bereits bewohntes Gespinst einzudringen, oder kommt sie auch nur mit den zahlreichen Gespinstfäden in Berührung, die das Gewebe an den Pflanzenteilen verankern, so reagiert die Gespinstinhaberin sofort mit einer Orientierung ihres Vorderkörpers in Richtung der herannahenden Konkurrentin. Hierauf beginnt sie, durch die Konkurrentin sichtlich gereizt, mit dem Vorderkörper heftig gegen die Gespinstwand in Form von Auf- und Abbewegungen zu schlagen. Diese schlagartigen Bewegungen werden in bestimmten Abständen von einem »Laufen auf der Stelle« unterbrochen.

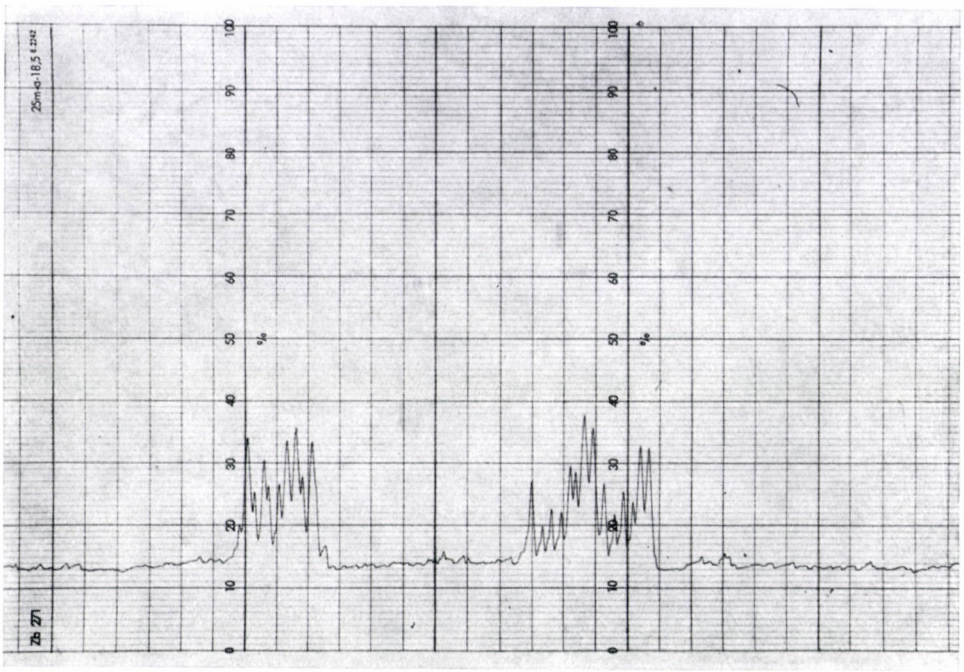

Abb. 1. »Klopfgeräusche« der Raupe von *Sparganothis pilleriana* Schiff. während einer Erregungsphase

Mit Hilfe der uns zur Verfügung stehenden bioakustischen Aufnahmetechnik war es möglich, diesen Reaktionsablauf auf Tonband aufzunehmen und hierauf mittels Servoschreiber graphisch zur Darstellung zu bringen (Abb. 1).

In Abb. 1 ist der Ablauf einer solchen Erregungsphase, im besonderen das Klopfgeräusch, zu erkennen. Jede Phase dieser Ausdrucksbewegung dauert etwa 3 bis 4 Sekunden und beinhaltet je nach Stärke der Reizung 13 bis 19 Schläge. In vielen Fällen konnte beobachtet werden, daß arteigene Raupen, die versuchten, das bewohnte Gespinst zu okkupieren, schon allein durch die Darbietung dieses »Klopfgeräusches« davon abgehalten wurden. Der Eindringling verhält sich dann vorerst ruhig und zieht sich hierauf ohne Kontakt mit der im Gespinst sitzenden Raupe gehabt zu haben, zurück.

Man wird nicht fehlgehen, wenn man annimmt, daß der durch das »Klopfen« erzeugte Erschütterungsreiz vom Eindringling mittels seiner zahlreichen Sinneshaare registriert und in seiner Bedeutung »verstanden« wird.

Versucht ein Eindringling trotz Darbietung des Erschütterungsreizes dennoch, in das bewohnte Gespinst einzudringen, so bewegt sich die Gespinstinhaberin energisch mit geöffneten Mandibeln in Richtung des Konkurrenten. Hierauf kommt es oft zu sehr heftigen Zweikämpfen, in deren Verlauf eine der Raupen

aus dem Gespinst getrieben wird. In keinem der bisher beobachteten Kämpfe waren die Tötung oder eine Verletzung eines der Partner zu beobachten.

b) *Pandemis ribeana* Hb. bewohnt zahlreiche Laubgehölze und Sträucher. Im besonderen konnten in Österreich in letzter Zeit starke Schäden an Johannisbeeren beobachtet werden. Ähnlich wie *Sparganothis pilleriana* Schiff. besiedeln auch die Raupen von *Pandemis ribeana* Hb. jeweils nur in Einzahl die Triebspitzen der Pflanzen und stellen auch Gespinste her.

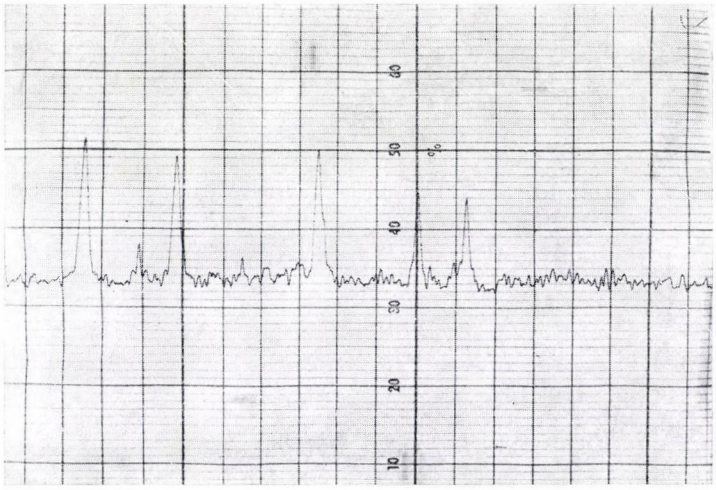

Abb. 2. »Klopfgeräusche« der Raupe von *Pandemis ribeana* Hb. während einer Erregungsphase

Laboratoriumsversuche mit Hilfe der bioakustischen Aufnahmegeräte zeigten auch bei *Pandemis ribeana* Hb. das Vorliegen eines Territorialverhaltens. Die graphische Darstellung der Beobachtungen erfolgte in Abb. 2.

Wie Abb. 2 erkennen läßt, bestehen offensichtlich in der Art der Darbietung des für *Pandemis ribeana* Hb. typischen »Klopfgeräusches« mit *Sparganothis pilleriana* Schiff. große Ähnlichkeiten. Eine genaue Analyse zeigt jedoch, daß wohl prinzipiell die Ausdruckbewegungen ebenfalls aus einem kräftigen Auf- und Abwärtsschlagen des Vorderkörpers bestehen, die zeitliche Folge der Schläge jedoch wesentlich gedehnter erfolgt. *Pandemis ribeana* Hb. bietet ihre Schläge nur in kräftigen Einzelschlägen dar und verbindet sie mit ausgedehnten Intervallen in Form eines »Laufens auf der Stelle«. Was den Effekt dieser Ausdrucksbewegung anbelangt, so ist dieser dem bei den Raupen von *Sparganothis pilleriana* Schiff. boebachteten vergleichbar.

c) *Chimabacche phryganella* Hb. ist in Österreich an Obstgehölzen relativ selten anzutreffen. Hingegen tritt die Art beispielsweise in Holland (mündl.

Mitteilung von De Jong, 1970) an Obstgehölzen, besonders an Apfelbäumen, sehr stark schädigend auf. Die Raupen dieser Art stellen ebenfalls ein sehr typisches Gespinst her, und zwar durch das Aneinanderspinnen zweier Blätter. Dieses Gespinst stellt ebenfalls das Freßterritorium der Art dar.

Was das territoriale Verhalten dieser Art anbelangt, so ergaben unsere diesbezüglichen Untersuchungen folgende interessanten Ergebnisse:

Die Raupen erzeugen mit Hilfe einer besonders stark entwickelten Kralle des metathorakalen Tarsus durch Scharren an der Unterlage des Gespinstes ein singendes Geräusch. (Siehe Abb. 3.) Bei Störung der Raupen durch intra- oder interspezifische Konkurrenten wird dieses Geräusch regelmäßig dargeboten. Mit Hilfe der bioakustischen Aufnahmetechnik war es möglich, dieses Geräusch akustisch aufzuzeichnen. Infolge zu starker Nebengeräusche gelang es jedoch nicht dieses Geräusch mit Hilfe des Servoschreibers auch bildlich darzustellen.

Im Gegensatz zu *Sparganothis pilleriana* Schiff. und *Pandemis ribeana* Hb. vollführt *Chimabacche phryganella* Hb. keine Auf- und Abbewegungen mit dem Vorderkörper, sondern bewegt in raschem Tempo die Thorakalbeine über die Blattfläche des Gespinstes, wobei besonders die metathorakale Tarsalkralle das typische, singende Geräusch erzeugt. Es handelt sich demnach bei dieser Ausdruckbewegung um ein besonders ausgeprägtes »Laufen auf der Stelle«, wozu gewissermaßen in dem stark verlängerten, metathorakalen Beinpaar ein eigenes geräusch- oder erschütterungerzeugendes Organ zur Verfügung steht. Zweifellos dient auch diese Form eines Erschütterungsreizes als territoriales Verteidigungsmittel.

Diskussion

Bioakustische Untersuchungen an Raupen von *Sparganothis pilleriana* Schiff., *Pandemis ribeana* Hb. und *Chimabacche phryganella* Hb. ließen erkennen, daß verschiedene, wenn nicht vielleicht sogar alle »solitär« lebenden Mikrolepidopteren mit Gespinstbau ein sehr ausgeprägtes Territorialverteidigungsverhalten besitzen. Dieses Verhalten dient vor allem dazu, das Freßterritorium gegen Eindringlinge (intra- oder interspezifischer Art) zu verteidigen. Dabei wird im besonderen der Erschütterungsreiz als Ausdrucksmittel verwendet. Bei den untersuchten Arten werden schlagende oder scharrende Bewegungen mit Hilfe des Lokomotionsapparates ausgeführt, die möglicherweise artspezifisch sind. Nach den bisherigen, allerdings vorerst noch wenigen Untersuchungen, wäre es denkbar, daß diese Ausdrucksbewegungen von Artgenossen in ihrer Bedeutung »verstanden« werden.

Phyllogenetisch dürfte die Entstehung solcher Ausdrucksbewegungen aus der Laufbewegung abzuleiten sein, da ja stets der Lokomotionsapparat zur Erzeugung der Erschütterungsreize verwendet wird. Ganz besonders fällt dies bei *Chimabacche phryganella* Hb. auf. Bei dieser Art wurde das metathorakale Beinpaar zu einem eigenen Ausdrucksorgan umgewandelt.

Es darf angenommen werden, daß sich dieses Territorialverhalten aus Flucht- und Aggressionsappetenz zusammensetzt und sich gewissermaßen als Resultante aus beiden Appetenzen als »Laufen an der Stelle« manifestiert und bei manchen Arten zu dem beobachteten Klopfen führt. Die Populationsdichte solcher Arten wird durch ein derartiges Territorialverhalten sicherlich sehr wesentlich beeinflußt. Eine ständige Abgrenzung und Verteidigung des Freßterritoriums, beispielsweise an den Triebspitzen, wird einer übermäßigen Dichtezunahme stets entgegenwirken und somit zu einer gleichmäßigen Distribution der Art auf der Wirtspflanze und im Wohngebiet führen.

Anderseits zeigten Versuche, die mit *Sparganothis pilleriana* Schiff. auf künstlichem Nährmedium ausgeführt wurden, daß in einem solchen Falle eine Dichtezunahme eher möglich ist. Trotzdem sollte die Bedeutung des Territorialverhaltens für Massenzuchten von Insekten nicht unterschätzt werden.

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Ecological Studies on the Arthropod Fauna of certain Libyan Soils

By

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The authors present the results of their investigations in sandy soils of Tripoli, Tagurah and in terra rossa soils of the El Marj and Shtata valleys, with special reference to soils of cultivated fields (vegetable plantations, cereals, orchards). Beside the qualitative and quantitative data of various groups and orders of animals found in these soils, the paper gives also data on the physical properties of the soils, on the climatic conditions and plant cover of the studied areas.

Soil animals are considered to be those which live permanently or temporarily in the soil and make an essential contribution to its formation. Almost every acre of soil is the home of many millions of insects, mites and other members of the phylum *Arthropoda*. According to MURPHY (1955), 96% of the arthropod population occurs in the first $2\,^1/_2$ inches of forest soils.

Several factors are known to affect the types and relative abundance of soil animal fauna. Among these factors which are of utmost importance are the physico-chemical properties of the soil, the plants cultivated on such soils, and agricultural practices (SCHIMITSCHEK, 1953; KEVAN, 1955; HAMMAD, 1960; KUHNELT, 1961; NASSAR, 1963; and EL-KIFL, 1965).

This review summarises the populations of arthropods found in varicus Libyan soils under changing climatic conditions and different plant covers.

Materials and Methods

Soil samples were obtained from the Universisty of Libya Agricultural Experimental Farm (Tripoli), Minis. of Agric., and Agrarian Reform Exp. Farm (Tripoli), a date-palm orchard and alfalfa field (Tagurah, 15 km east of Tripoli), Shtata Valley (20 km east of El-Marj) and a farm in El-Marj itself (1170 km east of Tripoli). The average degrees of temperature, precipitation and other information of both Tripoli and El-Marj regions are presented in Table 1 (after HAJJAJI, 1967). Weeds collected from the studied locations are shown in Tables 2 and 3.

The important physical properties of soil samples which were dealt with in the present review included: moisture content (%), water holding capacity (vol. %), bulk density, particle density, porosity (vol. %) and organic matter % (loss

Table 1

The average degrees of temperatures and precipitation throughout the year for El-Marj (av. of 70 years) and Tripoli (av. of 23 years) areas

	Temperature (°F.)		Precipitation (in inches)	
	El-Marj	Tripoli	El-Marj	Tripoli
January	50.9	53.4	4.62	3.03
February	52.0	55.9	3.42	1.66
March	55.2	59.4	1.66	0.97
April	60.8	64.6	0.67	0.38
May	64.4	69.1	0.24	0.20
June	73.0	74.8	0.05	0.06
July	74.7	78.1	0.01	0.02
August	74.8	79.3	0.02	0.03
September	73.0	77.9	0.08	0.39
October	68.4	72.3	1.43	1.42
November	61.5	64.8	2.23	2.66
December	54.1	56.3	4.67	3.76
Annual	63.9	67.1	19.08	14.50
Elevation (in feet)	935.0	56.0		
Latitude (in degrees and minutes)	32.40	32.54		
Longitude (in degrees and minutes)	20 54	13.11		

Table 2

Weeds collected in the orchards investigated

Fruit tree	Weed
Orange	*Euphorbia peptus*, *E. terracina*, *Emex spinosus*, *Bromus rigidus*, *Brussica tournefortii*, *Plantago lagopus*, *Launaea resedifolia*, *Medicago* sp., *Silena* sp., *Cyperus rotundus*, *Cynodon dactylon*, *Tripulu* sp., *Urospermum picroides*, *Urtica urons*
Olive	*E. terracina*, *Senecia desfontainei*, *Emex spinosus*, *Plantago lagopus*, *B. tournefortii*, *Imperata cylindrica*
Grape	*S. desfontainei*, *S. coronopifolius*, *Hippocrepis cyclocarpa*, *Daucus* sp.
Plum	*E. terracina*, *S. desfontainei*, *E. spinosus*, *Ononius serrata*, *Vicia calcarata*, *Medicago* sp., *Bromus* sp., *Agrotis stoloniferous*
Date-palm	*Medicago sativa*, *E. terracina*, *E. peplus*, *Cardnus* sp., *S. desfontainei*, *C. dactylon*, *Sataria* sp., *Erigeron* sp., *P. lagopus*, *C. rotundus*
Almond	*Avena* sp., *Malva parviflora*, *E. terracina*, *Sisymbrium irio*, *Sonchus oleraceus*, *B. tournefortii*

Table 3

The wild flora at the different heights of the hill

Base of the hill	Station No. 1 (15 m high)	Station No. 2 (30 m high)	Station No. 3 (45 m high)	Station No. 4 (60 m high)
1. *Hordeum* sp.	1. *Anagalis arvensis*	1. *A. arvensis*	1. *A. arvensis*	1. *Bromus rigidus*
2. *Trifolium* sp.	2. *Avena* sp.	2. *Bromus* sp.	2. *Hordeum inurinum*	2. *Trifolium campestre*
3. Compositae	3. *Koeleria* sp.	3. *Erodium* sp.	3. *Echium servecum*	3. *Trifolium angastifolium*
	4. *Anthenus* sp.	4. *Phlomis floccosa*	4. *Borago officinalis*	4. *Calendula microcanther*
	5. Compositae		5. *Sangiusorba verrucosa*	5. *Mercurialis annua*
			6. *Scabiosa* sp.	6. *Avena* sp.
				7. Two Compositae
				8. Two Umbelliferae
				9. Labiatae

by ignition). These were determined according to PIPER (1950) and CHAPMAN and PRATT (1961). For the extraction of soil arthropods, two soil samples were taken from every studied location by means of metal cylinders (one litre capacity and 15 cm deep). Extraction was carried out using the Tullgren-funnel method (TRAGARDH, 1928). Extraction period lasted about 48 hours for the light sandy soils of Tripoli and Tagurah and 72 hours for the heavier terra-rossa soil of Shtata Valley and El-Marj.

The soils in Tripoli and Tagurah are of light colour, almost of homogeneous, fine sand particles, with about 30–38 water holding capacity (vol. %) and contain 1.91–2.98% organic matter (Tables 4, 5, 6, 7, 9, and 10). On the other hand soils of El-Marj and Shtata Valley are reddish in colour, generally called "Hamra" earth or "Terra-rossa", homogeneous, compact, with about 44–50% water holding capacity and contain 2.8–3.8% organic matter (Tables 4 and 8).

Results

In all the investigated Libyan soils, acarina formed the highest percentage of the total arthropod fauna. The numbers of trombidiform mites were mostly higher in the light sandy soils of Tripolitania than those of oribatid mites (Tables 5, 6, and 7). Those latter mites dominated in El-Marj and Shtata Valley soils which are rich in the organic matter (Tables 4, 7, and 8). Mesostigmatid mites over-numbered the trombidiform mites in most cases in the light sandy soils (Ta-

Table 4

Physical properties of tested barley soils (Shtata Valley, El-Marj, and Tripoli areas)

Physical property	Shtata Valley	El-Marj	Tripoli (Univ. Farm)
Moisture (%)	4.93	8.20	2.60
Water holding capacity (vol. %)	48.60	44.0	40.0
Vol./100 gm (cc.)	79.30	80.0	72.0
Bulk density	1.26	1.25	1.38
Particle density	2.38	2.38	2.27
Porosity (vol. %)	47.20	47.50	39.30
Organic matter (%)	3.26	3.80	2.56
pH	6.0	6.5	7.5

Numbers of Arthropoda in the barley soils (in one litre soil and 15 cm deep)

Fauna	Shtata Valley		El-Marj		Tripoli	
	Mean	%	Mean	%	Mean	%
Pauropoda	0.30	0.20	—	—	—	—
Chilopoda	0.67	0.37	—	—	—	—
Diplopoda	0.33	0.20	—	—	—	—
Mesostigmata	5.33	2.72	1	0.79	2.5	16.10
Trombidiformes	12.66	6.82	15	11.83	0.5	3.22
Oribatei	158.60	81.70	105	82.84	0.5	3.22
Total mites	176.50	66.91	239	95.50	3.5	22.54
Collembola	2.33	1.19	—	—	4.0	25.76
Psocoptera	0.33	0.17	1	0.79	4.0	25.76
Thysanoptera	4.66	2.40	—	—	1.5	9.66
Hemiptera	1.33	0.71	—	—	0.5	3.22
Coleoptera	2.66	1.57	4	3.16	0.5	3.22
Diptera	2.33	1.99	2	1.58	0.5	3.22
Hymenoptera	0.33	0.17	—	—	0.5	3.22
Total Arthropoda	192.0	100.0	128	100.0	15.0	100.0

bles 4, 7, and 8). This agrees with the findings of LOOTS and RYKE (1967) and NASSAR (1963). Large numbers of *Acaridiae* were only found in soils under tomato (Table 7). *Collembola* increased with the increase of soil moisture (Tables 4, 6, and 7). Members of the insect orders *Psocoptera*, *Thysanoptera*, *Hemiptera*, *Homoptera*, *Coleoptera*, *Diptera*, and *Hymenoptera* were represented each by a few number (Tables 4 to 10).

Terra-rossa soils of Shtata Valley and El-Marj contained higher arthropod counts than the light-sandy soils of Tripoli (Table 4). This is due to the higher amounts of organic matter and the higher water holding capacity in the Terra-

Table 5

Physical properties of soil samples (Univ. Farm, Tripoli)

Physical property	Wheat	Broad bean	Alfalfa
Moisture (%)	3.56	4.12	5.40
Water holding capacity (vol. %)	38.0	38.0	40.0
Bulk density	1.33	1.31	1.35
Particle density	2.52	2.27	2.22
Porosity	47.30	42.30	30.50
Organic matter (%)	2.60	2.466	2.580

Numbers of soil animal fauna (in one litre soil and 15 cm deep)

Fauna	Wheat		Broad bean		Alfalfa	
	Mean	%	Mean	%	Mean	%
Annelida (Enchyt.)	0.5	0.59	2.5	19.23	—	—
Mollusca	—	—	—	—	0.5	0.57
Mesostigmata	1.5	4.47	3.0	23.08	80.0	91.20
Trombidiformes	1.5	4.47	0.5	3.85	1.0	1.14
Oribatei	21.5	68.24	0.5	3.85	0.5	0.57
Total mites	24.5	77.76	4.0	30.77	81.5	92.91
Collembola	1.0	3.17	1.5	11.54	1.5	1.71
Psocoptera	0.5	1.59	—	—	—	—
Thysanoptera	2.0	6.35	0.5	3.85	—	—
Homoptera	—	—	—	—	1.0	1.14
Lepidoptera	—	—	—	—	0.5	0.57
Coleoptera	2.0	6.33	2.0	15.38	1.5	1.71
Diptera	—	—	2.5	19.23	0.5	0.57
Hymenoptera	1.0	3.17	—	—	0.5	0.57
Total Arthropoda	31.0	98.41	10.5	80.77	87.0	99.18
Total fauna	31.5	100.0	13.0	100.0	87.5	100.0

rossa soils. Members of *Pauropoda*, *Chilopoda*, *Diplopoda*, *Symphyla*, *Protura* and *Thysanura* were only represented in the Terra-rossa soils (Tables 4 and 8), while they were absent from all the tested light sandy soils of Tripoli except those under almond trees which harboured members of *Crustacea (Isopoda)*, *Chilopoda*, and *Symphyla*. NASSAR (1963), working on the animal fauna of different types of Egyptian soils, found those latter arthropod groups in soils relatively rich in organic matter.

The plant cover had an effect on the arthropod populations. Soils under vegetable crops generally contained the highest numbers, followed by soils under fruit trees and then soils under field crops. From the vegetable crops, tomato and

Table 6

Physical properties of soil samples under fruit trees (Univ. Farm, Tripoli)

Physical property	Orange		Olive		Grape		Plum	
	Feb.	June	Feb.	June	Feb.	June	Feb.	June
Moisture (%)	2.59	2.31	1.33	1.29	2.19	1.89	1.16	1.42
Water holding capacity (vol. %)	36	36	30	31	34	35	36	36
Volume/100 gm (cc)	78	78	72	72	76	76	74	74
Bulk density	1.28	1.28	1.38	1.38	1.31	1.30	1.35	1.35
Particle density	2.80	2.80	2.80	2.80	2.77	2.75	2.70	2.72
Porosity (vol. %)	45.70	45.70	49.30	49.30	47.30	47.60	50.00	49.60
Organic matter (%)	2.59	2.43	2.13	2.06	2.20	2.15	2.98	2.67

Mean individual numbers of soil animal fauna (in one litre soil and 15 cm deep

Fauna	Orange		Olive		Grape		Plum	
	Feb.	June	Feb.	June	Feb.	June	Feb.	June
Mollusca	22.0	44.0	10.0	11.0	0.5	1.0	3.5	11.0
Araneae	—	—	—	—	—	—	0.5	—
Mesostigmata	12.0	—	0.5	—	—	—	2.5	—
Trombidiformes	2.5	20.0	1.5	7.5	—	24.0	2.5	5.0
Oribatei	19.0	18.0	12.0	10.5	3.0	20.0	5.0	0.5
Total mites	33.5	38.0	14.0	18.0	3.0	44.0	9.0	5.5
Collembola	—	34.5	—	—	—	8.0	2.0	1.0
Psocoptera	0.5	—	3.0	—	—	2.0	0.5	1.0
Thysanoptera	0.5	—	—	—	—	—	9.5	—
Hemiptera	—	—	—	—	—	—	0.5	—
Homoptera	—	0.5	—	1.0	—	1.5	—	0.5
Coleoptera	1.0	0.5	—	—	—	—	4.0	—
Diptera	2.0	—	3.0	—	0.5	0.5	1.0	—
Hymenoptera	—	—	0.5	—	—	—	0.5	0.5
Total Arthropoda	39.5	73.5	20.5	19.0	3.5	58.5	21.0	8.5

artichoke soils harboured the highest numbers, followed by carrot, then pea, then raddish and the least was lettuce (Table 7). Soils under date-palms were also the richest in their arthropod populations compared with other investigated fruit soils, followed by soils under almond trees, then soils under grape, followed by soils under olive and the least in their arthropod populations were soils under plum trees (Tables 8, 9, and 10). As far as the field crops as plant covers are concerned, alfalfa soils harboured the highest numbers of arthropods, followed by wheat soils, then barley soils and the least arthropod population was found in broad bean soils.

Table 7

Physical properties of the vegetable soils studied (Minis. Agric. and Agric. Reform Exp. Farm, Tripoli)

Physical property	Tomato	Artichoke	Pea	Carrot	Raddish	Lettuce
Moisture (%)	2.4	2.49	3.02	3.68	3.70	3.06
Water holding capacity (vol. %)	36.0	36.0	36.0	36.0	36.0	36.0
Bulk density	1.38	1.39	1.38	1.38	1.38	1.38
Particle density	2.70	2.73	2.70	2.71	2.72	2.70
Porosity	51.11	50.91	51.11	50.99	50.73	51.11
Organic matter (%)	2.12	2.03	2.51	2.51	2.51	2.51

Numbers of soil arthropods in one litre soil and 15 cm deep in the same tested vegetable soils

Fauna	Tomato		Artichoke		Pea	
	Mean	%	Mean	%	Mean	%
Trombidiformes	48.5	10.77	24.5	7.25	4.5	10.71
Mesostigmata	43.0	9.55	52.0	15.39	16.0	38.08
Oribatei	88.0	19.54	78.5	23.24	12.0	28.56
Acaridiae	179.0	39.73	—	—	—	—
Total mites	363.5	80.70	155.0	45.88	32.5	77.35
Collembola	75.5	16.76	18.0	53.58	7.5	17.85
Psocoptera	0.5	0.11	—	—	—	—
Coleoptera	7.0	1.56	2.0	0.59	0.5	1.19
Diptera	3.5	0.78	—	—	1.0	2.38
Hymenoptera	—	—	—	—	0.5	1.19
Total arthropoda	450.0	100.0	330.0	100.0	42.0	100.0

Fauna	Carrot		Raddish		Lettuce	
	Mean	%	Mean	%	Mean	%
Trombidiformes	14.0	20.27	2.0	11.43	1.0	20.0
Mesostigmata	20.0	28.96	7.0	40.0	2.0	40.0
Oribatei	34.0	49.23	7.0	40.0	1.5	30.0
Acaridiae	—	—	—	—	—	—
Total mites	68.0	98.46	16.0	91.42	4.5	90.0
Collembola	—	—	—	—	—	—
Psocoptera	—	—	0.5	2.86	—	—
Coleoptera	0.5	0.72	0.5	2.86	—	—
Diptera	0.5	0.72	0.5	2.86	0.5	100.0
Hymenoptera	—	—	—	—	—	—
Total arthropoda	69.0	100.0	17.5	100.0	5.0	100.0

Table 8

Physical properties of soil samples (at different altitudes of a hill (El-Marj)

Physical property	Base	15 m high	30 m high	45 m high	60 m high
Moisture (%)	8.20	9.0	7.60	8.80	10.60
Water holding capacity (vol. %)	36.0	36.0	36.0	36.0	32.0
Volume per 100 gm (cc)	86.0	90.0	92.0	86.0	88.0
Bulk density	1.16	1.11	1.09	1.16	1.14
Particle density	2.43	2.38	2.38	2.32	2.38
Porosity (%)	52.20	53.40	54.40	50.0	52.30
Organic matter (%)	3.10	5.20	5.30	5.0	5.30
pH	7.0	6.5	6.5	6.5	6.0

Numbers of soil arthropods (in one litre soil and 15 cm deep)

Fauna	Base		15 m.		30 m.		45 m.		60 m.	
	Mean	%	Mean	%	Mean	%	Mean	%	Mean	%
Pseudoscorpionida	—	—	1.0	—	—	—	—	—	—	—
Pauropoda	—	—	1.0	0.26	1.0	0.46	—	—	3.0	0.79
Chilopoda	1.0	0.11	—	—	—	—	—	—	—	—
Diplopoda	—	—	—	—	—	—	—	—	3.0	0.79
Symphyla	1.0	0.11	—	—	—	—	—	—	—	—
Mesostigmata	20.0	2.18	115.0	30.47	70.0	32.20	70.0	11.48	194.0	51.41
Trombidiformes	5.0	0.54	17.0	4.50	50.0	23.0	414.0	67.90	12.0	3.18
Oribatei	50.0	5.45	230.0	40.95	83.0	83.18	100.0	16.4	92.0	24.38
Total mites	75.0	8.17	362.0	75.93	203.0	93.38	584.0	95.78	298.0	78.97
Protura	—	—	—	—	—	—	6.0	0.98	9.0	2.38
Collembola	5.0	0.54	11.0	2.91	10.0	4.60	19.0	3.12	31.0	8.21
Thysanura	1.0	0.11	—	—	—	—	1.0	0.16	2.0	0.53
Psocoptera	2.0	0.22	—	—	—	—	2.0	0.33	—	—
Thysanoptera	3.0	0.33	—	—	—	—	—	—	2.0	0.53
Homoptera	—	—	1.0	0.26	3.0	1.38	—	—	—	—
Coleoptera	2.0	0.22	2.0	0.53	—	—	—	—	25.0	6.62
Diptera	—	—	—	—	—	—	2.0	0.33	4.0	1.26
Hymenoptera	1.0	0.11	—	—	—	—	—	—	—	—
Total arthropoda	91.0	100.0	377.0	100.0	217.0	100.0	608.0	100.0	377.0	100.0

Members of *Enchytridae (Annelida)* were found in few numbers in sandy soils under wheat, broad bean during February (Table 3) and date-palm during November and January (Table 9) and in large numbers in similar sandy soils but under almond trees during March (Table 10). No Lumbricid Annelida were found in all the examined samples of the Libyan soils, which agrees with the findings of

Table 9

Physical properties of soil samples in a date-palm alfalfa orchard (Tagurah)

Physical property	June 1969	Nov. 1969	Jan. 1970	March 1970
Moisture (%)	2.42	2.54	2.91	2.86
Water holding capacity (vol. %)	37.0	37.0	37.0	37.0
Bulk density	1.37	1.36	1.38	1.38
Particle density	2.72	2.72	2.72	2.72
Porosity (%)	50.40	50.0	50.70	50.70
Organic matter (%)	2.16	2.30	2.23	2.40

Numbers of soil animal fauna (in one litre soil and 15 cm deep) in a date-palm alfalfa orchard (Tajurah)

Fauna	June, 1969		Nov., 1969		Jan., 1970		March, 1970	
	Mean	%	Mean	%	Mean	%	Mean	%
Annelida	—	—	4.5	—	0.5	—	—	—
Aranea	—	—	—	—	—	—	1.5	1.14
Mesostigmata	62.5	34.38	81.0	21.87	36.5	28.84	56.5	42.83
Trombidiformes	13.0	7.15	15.5	4.19	0.5	0.40	8.0	6.06
Oribatei	34.5	18.98	271.5	73.31	82.0	64.78	46.0	34.87
Total mites	110	60.5	368	99.36	119	94.01	110.5	83.76
Collembola	17.0	9.35	1.0	0.28	5.5	4.35	—	—
Psocoptera	—	—	—	—	1.0	0.79	0.5	0.38
Thysanoptera	—	—	—	—	—	—	0.5	0.38
Hemiptera	—	—	—	—	—	—	1.5	1.14
Homoptera	51.5	27.88	—	—	—	—	5.5	4.18
Coleoptera	2.5	1.37	—	—	0.5	0.40	6.0	4.56
Diptera	0.5	0.27	1.0	0.28	0.5	0.40	6.0	4.56
Total Arthropoda	181.5	100.0	370	100.0	126.5	100.0	132	100.0

NASSAR (1963) who did not find *Lumbricid* worms in saline, alkaline and sandy soils of Egypt.

Theba pisans MULLER (Mollusca), which is a serious agricultural pest in the coastal regions of Libya, showed host preference. It was found in a few number in soils under barley, alfalfa, grape and plum, in considerable numbers in soils under olive and almond trees and in large numbers in soils under orange trees.

The counts of soil arthropods at different heights of a hill at El-Marj region showed that arthropod fauna were different in their densities and categories at the different altitudes (Table 8). The hill is about 80 m high, its base is about 400 m above sea-level, and made up mostly of limestone, both calcareous and

Table 10

Physical properties of soil samples under almond trees (Univ. Farm, Tripoli)

Physical property	June 1969	Nov. 1969	Jan. 1970	March 1970
Moisture (%)	1.22	2.06	2.61	1.98
Water holding capacity (vol. %)	36.0	36.0	36.0	36.0
Bulk density	1.38	1.37	1.37	1.37
Particle density	2.70	2.70	2.70	2.70
Porosity (%)	49.50	50.70	50.70	50.70
Organic matter (%)	1.906	2.180	2.120	1.930

Numbers of soil animal fauna (in one litre soil and 15 cm deep) under the same almond trees

Fauna	June 1969		Nov. 1969		Jan. 1970		March 1970	
	Mean	%	Mean	%	Mean	%	Mean	%
Annelida	—	—	1.5	—	4.0	—	102.5	—
Mollusca	11.0	—	—	—	—	—	—	—
Crustacea (Isopoda)	—	—	—	—	0.5	0.83	—	—
Chilopoda	—	—	0.5	20.0	—	—	—	—
Symphyla	—	—	—	—	4.0	6.66	6.0	6.14
Araneae	—	—	—	—		—	1.0	1.02
Mesostigmata	1.5	6.52	0.5	20.0	7.5	12.49	23.0	23.55
Trombidiformes	5.5	23.91	0.5	20.0	18.5	30.82	8.5	8.70
Oribatei	7.5	32.61	0.5	20.0	15.0	24.99	18.0	18.43
Total mites	14.5	63.04	1.5	60.0	41.0	78.30	49.5	50.69
Collembola	1.5	6.52	0.5	20.0	9.0	14.99	23.0	23.55
Psocoptera	6.0	26.09	—	—	—	—	0.5	0.51
Homoptera	0.5	2.17	—	—	1.0	1.66	2.5	2.57
Coleoptera	—	—	—	—	3.5	5.83	3.5	3.58
Diptera	0.5	2.17	—	—	1.0	1.66	11.5	11.77
Total Arthropoda	23.0	100.0	2.5	100.0	60.0	100.0	97.5	100.0

foraminiferous. Soil samples were taken at the base and then at 15 m, 30 m, 45 m, and 60 m high, respectively. A regular increase in the total arthropod counts was noticed with the increase of altitude.

The numbers and categories of soil arthropods change from season to season due to the prevailing weather conditions. In Tripoli region, numbers of arthropods were estimated in soils under orange, grape, olive, and plum in February and June (Table 6) and in soils of date-palm-alfalfa (Table 9) and almond orchards (Table 10) in June, November, January and March. Generally, arthro-

pod populations under orange, grape, olive and plum were lower in June than in February. *Collembola* disappeared in most samples in June, while coleopterous and dipterous insects appeared in June, but they were absent in February. This agrees with the findings of MADGE (1969) who concluded that number of invertebrates decreases intensively in the dry season as compared to the wet season in forest and savanna soils. In the soil of a date-palm orchard at Tagurah, which was regularly irrigated every 15 days, the total arthropod counts were highest in November, followed by June, and low in March and January. Counts of these last two months were almost the same. *Collembola* were absent in March, few in November, relatively higher in number in January and rather high in June. *Homoptera* (Aphids) appeared first in March and attained their maximum number in June. Coleopterous and dipterous insects were poorly represented, although of more occurrence in March. MOURSI et al. (1966) recorded the highest counts of arthropods in soil of a date-palm citrus orchard in Iraq in autumn, which agrees with the results obtained in the present work. In soil of the almond orchard, the numbers of arthropods in general and acarina in particular were very low in November. *Collembola* were represented by a few individuals in June, a fairly high number in January and a very high count in March. Members of other insect orders were found in few numbers in January and March. This shows that not only the prevailing weather conditions affect the numbers and categories of arthropods in Tripoli soils, but also other factors such as the plant cover and soil conditions have an effect on these numbers and categories of soil arthropods.

*

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Das Verhalten des Kartoffelkäfers (*Leptinotarsa decemlineata* Say) gegenüber drei Kartoffelsorten

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In the experiments the development and survival of Colorado potato beetle (*Leptinotarsa decemlineata* SAY) were studied on three potato varieties, of whom two (Ackersegen, Bintje) came from France, the third (Kennebec) from Canada. In laboratory experiments the young plants of the variety "Kennebec" were less suitable for the development and reproduction of the pest as the ones of "Bintje". In field experiments the survival of larvae was studied as a function of feeding conditions of the preceding parental generation on the three varieties mentioned. According to these preliminary observations the variety "Kennebec" seems to contain some resistance factors; a rational, continuous "rotation of varieties" grown may result in a decrease of Colorado beetle population densities.

Die Nahrung, die Fruchtbarkeit und die Entwicklung des Kartoffelkäfers, *Leptinotarsa decemlineata* SAY *(Coleoptera Chrysomelidae)*, sind seit vielen Jahren (TROUVELOT, JERMY, GRISON, de WILDE) mit Rücksicht auf die Art oder die Sorte des angebotenen Kartoffels studiert worden.

Diese Untersuchungen sollten die Entdeckung oder die Züchtung von solchen Sorten ermöglichen, die eine Resistenz gegen diesen Käfer aufweisen.

Solanum Arten, die in verschiedenen Teilen Amerikas spontan wachsen, wie *Solanum demissum* oder *S. chacoense*, wurden am meisten studiert.

Gegenüber den in Frankreich augebauten Sorten verhält sich der Kartoffelkäfer verschieden. So wird die Vermehrung des Käfers durch »Ackersegen« mehr gefördert als durch »Bintje« (GRISON, 1963).

Die Sorte »Kennebec«, die im 1941 in den Vereinigten Staaten gezüchtet war, schien zur Vermehrung des Schädlings wenig geeignet zu sein, z. B. in Québec (Kanada) (JOBIN, 1967). Die vorliegende Mitteilung gibt die ersten Ergebnisse wieder, die in Versailles (Frankreich) beim Vergleich der drei Sorten »Kennebec«, »Bintje« und »Ackersegen« erhalten worden sind.

Laborversuche

Die Untersuchungen wurden bei einer Temperatur von 25 °C und einer Photoperiode von 16 Stunden durchgeführt.

Die genützten Käfer kommen aus einer Dauerzucht auf »Bintje« her.

a) *Topfversuche mit jungen Pflanzen*

Die vollständigen jungen Pflanzen werden eingeführt, wenn sie nur zwanzig Zentimeter hoch sind.

	Sorte »Bintje«	Sorte »Kennebec«
Anzahl von Larven L_1 (erstes Stadium)	55	84
Dauer der Stadien 1, 2, 3 ($L_1 + L_2 + L_3$)	6—7 Tage	8—9 Tage
Anzahl verkrochener Larven	34 (62%)	36 (42%)
Anzahl aufgetauchter Imagines	24 (43%)	12 (14%)
Gelege von 5 Paaren (6.—16. Tag)	721 ω	279 ω

So erwies sich unter diesen Verhältnissen die Sorte »Kennebec« weniger günstig für die Käfer als »Bintje«: die Larven entwickeln sich langsamer und schlechter und die Imagines legen weniger Eier.

b) *Versuch mit abgeschnittenen Blättern und Zweigen*

In diesem Versuch werden die Käfer mit abgeschnittenen Blättern und Zweigen ernährt, die täglich erneut wurden.

	Sorte »Bintje«	Sorte »Kennebec«
Anzahl der untersuchten L_1 Larven	102	196
Dauer der Stadien 1, 2, 3	5—6 Tage	5—7 Tage
Anzahl verkrochener Larven	46 (45%)	129 (66%)
Anzahl aufgetauchter Imagines	27 (26%)	104 (53%)
Durchschnittl. Lebensdauer des Imagines	42 Tage	27 Tage
Durschchnittl. Eiablage pro Weibchen mit Lebensdauer über eine Woche	615 ω	208 ω

Dabei wird ersichtlich, daß die Sorte »Kennebec« ihre Resistenzfähigkeit gegen Larven verliert, wenn sie als abgeschnittene Blätter den Larven gegeben sind. Doch bleibt ihre Einfluß auf die Fruchtbarkeit der Imagines erhalten. Anderseits ist die Lebensdauer der Käfer kürzer auf »Kennebec« als auf «Bintje«.

Die Abschwächung der Resistenzfähigkeit bei den abgeschnittenen Teilen im Vergleich zu vollständigen Pflanzen und bei alten Pflanzen im Vergleich zu jungen Pflanzen ist eine schon bekannte Erscheinung (Le Berre 1967).

Feldversuche

Das Kartoffelpflanzgut der Sorten »Ackersegen«, »Bintje« und »Kennebec« wurde am 5. Mai eingestellt; 36 Sämlinge je Sorte wurden abwechselnd in derselben Parzelle angeordnet, so daß sie insgesamt 108 Pflanzen enthielt.

Um einen einheitlichen Befall zu erreichen, wurde die Verseuchung mit Gelege und Larven im ersten Stadium durchgeführt. Das Sammeln der auf die Parzelle gelangten Imagines erlaubte, den sehr schwachen natürlichen Befall auszuschließen.

Die Eltern der genützten Eier und Larven wurden im Laboratorium teils auf Bintje-Blätter, teils auf Kennebec-Blätter angezüchtet.

Insgesamt 4638 Eier und Larven im ersten Stadium wurden von 9. Juni bis zum 27. Juli verteilt. Die Larven wurden in regelmäßigen Zeitspannen gezählt und auf jeder Pflanze gesammelt, als sie das vierte Stadium erreicht hatten. Die folgende Tabelle gibt die erhaltenen Ergebnisse wieder.

Eltern auf untenstehenden Sorten gezüchtet	Larvenentwicklung auf	Eier oder L_1	L_4 (%)
Bintje	Ackersegen	742	117 (15,8)
	Bintje	772	57 (7.4)
	Kennebec	774	124 (16.0)
Kennebec	Ackersegen	854	57 (6.7)
	Bintje	851	40 (4.7)
	Kennebec	645	44 (6.8)

Zwei besondere Erscheinungen können hervorgehoben werden: – die Zahl überlebender Larven ist geringer wenn die Eltern auf »Kennebec« gefüttert waren, als wenn sie sich auf »Bintje« ernähren – auf »Bintje« ist die Zahl überlebender Larven schwächer als auf den beiden anderen Sorten.

Es sei dabei erwähnt daß »Bintje« die frühreifste Sorte ist und daß sie kaum noch Schößlinge vom Juli an bildet, im Gegensatz zu der mäßig frühreifen »Kennebec« und vor allem zu der spätreifen »Ackersegen«.

Diskussion

Nach diesen Vorversuchen scheint die Sorte »Kennebec« in Frankreich gewisse Resistenzfaktoren gegen den Kartoffelkäfer aufzuweisen, z. B. wäre sie weniger anfällig als »Bintje«.

Es wird daran erinnert, daß der Anbau von Sorten mit einer relativen Resistenzfähigkeit die Vermehrung des Schädlings vermindern kann, insbesondere wenn frühreife Sorten genützt wurden, die keine richtige Nahrung für die Sommerimagines darbieten (GRISON, 1963).

Diese Vorversuche lassen dazu die Wirkung des »Sortenwechselspiels« auf die Beschränkung einer Vermehrung dieses Kartoffelschädlings vermuten.

Zusammenfassung

In unseren Experimenten wurde die Entwicklung und Überleben des Kartoffelkäfers (*Leptinotarsa decemlineata* SAY) an drei verschiedenen Kartoffelsorten untersucht, aus welchen zwei (Ackersegen und Bintje) aus Frankreich, eine (Kennebec) aus Kanada stammten.

In Laboratoriumsversuchen hatten sich die jungen Pflanzen der Sorte »Kennebec« weniger geeignet erwiesen für die Entwicklung und Vermehrung des Schädlings als die der Sorte »Bintje«.

In Feldversuchen wurde das Überleben der Larven als eine Folge der Ernährungsverhältnisse der vorigen Generation an den drei erwähnten Sorten untersucht. Nach diesen Vorversuchen scheint die Sorte »Kennebec« in Frankreich gewisse Resistenzfaktoren aufzuweisen. Ein gezielter, wiederholter Sortenwechsel könnte auf dieser Basis zu einer Beschränkung der Kartoffelkäferpopulation führen.

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The Effect of Parasites on a Population of *Pegomyia hyoscyami* Panzer

By

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During the period 1967—1969 studies were carried out in the Lublin and Warsaw voivodships, to which degree *Pegomyia hyoscyami* Panzer *s.l.* was attacked by parasites. Dissection and rearing of fly puparia produced 1.233 individuals of hymenopterous parasites, the majority of which belonged to the genus *Opius* Wesm. Of these *Opius spinaciae* Thoms. was dominant, making up 64.6% of all parasites obtained. Rearing of puparia obtained from mature larvae of *Pegomyia hyoscyami* Panz. (2.928 individuals) showed that the degree of invasion of the pest by parasites was on an average 39.4% in 1967—1968, and 22.3% in 1969. In many places the parasitization exceeded 60%. Analysis of dissected larvae (4.074 individuals) showed that the process of parasitization begins very early, during the first stages of larval development. Conditions in 1967 were favourable to the development of *Pegomyia hyoscyami* Panzer; the beet fly appeared in relative large numbers and produced 4 generations. In 1968 and 1969, however, the individual density was low and only 2 generations were produced.

The beet fly occurs in all parts of Poland as a pest frequently causing serious losses in beet crops. It is particularly in spring that the great abundance of larvae feeding on the plants often results in inhibited growth of the young beet plants. The damage done is more serious in years in which the spring is warm and dry, since such weather is favourable to the flight and egg-laying of this fly, and roots of these plants are still weak owing to low soil humidity.

Intensified occurrence of the beet fly depends on a large number of abiotic and biotic factors, including the degree to which the insect is invaded by parasites. The group of parasites may take different forms in different parts of the country depending on local conditions, and may alter depending on the generation of the beet fly. According to PAWELSKA and SANDNER (1962), ŘEHÁK (1966) and SKUHRAVÝ et al. (1967), warm dry weather towards the end of the beet's growing season encourages the development of parasites, whereas cool and wet summers create conditions unfavourable to them, as during the imago stage they are more susceptible to weather conditions than their host, the beet fly.

Studies were made from 1967—1969 on the role of parasites as a factor reducing population numbers of the beet fly. Observations were made in the Warsaw and Lublin voivodships on sugar and fodder beet plantations. During the height of the growing season weekly inspections were made of the plantations,

collecting eggs and larvae of the beet fly each time. Eggs and the mature larvae were reared, and the immature larvae dissected.

Laboratory culture of the eggs proved most successful, frequently obtaining 100% hatching. Hatching rate was very rapid; the greatest number of eggs hatched on the second and third day of culture. Eggs from which no larvae had hatched by the 7th day proved to be dead. A total of 16.467 eggs were taken for culture over the whole study period (Table 1). Despite the large number of eggs cultured none of them were found to be invaded by parasites, but the larvae of *Chrysopa vulgaris* Schn. were observed to feed on beet fly eggs. One larva of this predator placed in an isolator with 50 beet fly eggs, destroyed almost the entire culture within 24 hours. Larvae of *Ch. vulgaris* isolated with young beet fly larvae sucked the latter dry through the leaf epidermis.

Table 1

Results of rearing beet fly eggs

Year	Number of eggs cultured	Hatched larvae		Dead eggs	
		Number	%	Number	%
1967	15.607	15.065	96.5	542	3.5
1968	720	700	97.2	20	2.8
1969	140	136	97.1	4	2.9
Total	16.467	15.901	96.6	566	3.4

Field observations revealed a far slower hatching rate and far lower percentage of hatched eggs. The degree to which eggs are reduced by predators on the plantations was very difficult to define but there is considerable justification for assuming that 5–7% of the eggs were destroyed by unidentified predators.

Mature beet fly larvae found on the plantations in sections of mined leaves, were placed in dishes with a layer of soil on the bottom. After only a few hours the beet fly larvae left their feeding places and moved to the soil, where they formed puparia. The majority of the larvae formed puparia on the second and third day of rearing. A total of 3.373 beet fly larvae were taken for culture from 1967–1969, and 2.928 puparia obtained from them. These puparia were used for further culture in order to define the degree to which the pest was invaded by parasites. The first beet fly imagoes began to appear on the 12th–15th day after formation of puparia. The flight of parasitic *Hymenoptera* was from 10–15 days or more later than the flight of beet fly imagoes.

Culture of puparia yielded 645 beet fly imagoes and 1.019 parasitic *Hymenoptera* (Table 2). The average parasitization of the beet fly was high: 63.8% in 1967 and 71.3% in 1968. In 1969 invasion of the pest by parasites fell to 49.2%, probably due to the unfavourable weather during the flight period of the delicate parasitic *Hymenoptera*.

Table 2

Results of rearing beet fly puparia

Year	Number of puparia cultured	Number of imagines hatched from beet fly puparia					Number of dead puparia
		Total	Beet fly		Hymenoptera parasitica		
			Number	%	Number	%	
1967	1.893	1.170	424	36.2	746	63.8	723
1968	246	136	39	28.7	97	71.3	110
1969	789	358	182	50.8	176	49.2	431
Total	2.928	1.664	645	38.7	1.019	61.3	1.264

No emergence flights were obtained from 1.264 of the puparia cultured. On dissection the majority of these puparia were found to contain beet fly larvae which had died before pupation: their maceration or desiccation made it impossible to determine whether any parasite was present. In 42 puparia formed dead beet fly imagoes were found, and in 214 – parasitic *Hymenoptera* imagoes of the genus *Opius* Wesm., which were also dead. The results of analysis of the dissected puparia are given in Table 3. Dissection of the dead puparia showed that a far higher percentage of the beet flies were invaded by parasites than was the case in the cultures.

Table 3

Results of dissecting dead beet fly puparia

Year	Number of dissected puparia	Puparia with dead praepupae	Puparia with dead imagoes			
			Beet fly		Hymenoptera parasitica	
			Number	%	Number	%
1967	723	567	24	4.2	132	23.3
1968	110	67	11	16.4	32	47.8
1969	431	374	7	1.9	50	13.4
Total	1.264	1.008	42	4.2	214	21.2

A joint total of 1.233 individuals of parasitic *Hymenoptera* were obtained from culture and dissection of puparia. Among these species of the genus *Opius* predominated, the dominant being the species *O. spinaciae* THOMS. which gave 73.9% of all the parasites obtained (Table 4). The other species occurred in small numbers only, or even sporadically. MICZULSKI and PAWELSKA (1964) found a

Table 4

Hymenoptera parasitica of beet fly (from culture and dissection)

Species	Number of individuals				Percentage
	1967	1968	1969	jointly	
Opius fulvicollis THOMS.	54	28	13	95	7.70
Opuis rufipes WESM.	—	1	—	1	0.08
Opius ruficeps WESM.	6	2	4	12	0.97
Opius nitidulator NEES.	64	3	18	85	6.97
Opius spinaciae THOMS.	716	57	138	911	73.90
Opius sp.	47	6	38	91	7.30
Phenoserphus sp.	1	—	—	1	0.08
Phygadeuon sp.	16	—	1	17	1.40
Pseudeucoila sp.	18	—	2	20	1.60
Total	922	97	214	1.233	100.00

similar species composition for beet fly parasites, but the average parasitization of the pest found by these authors was far lower than the results we have presented here.

Analysis of 4.047 dissected larvae of the beet fly collected in different stages of growth showed that invasion by parasites begins very early, in growth stage I (L_1), but mature larvae of growth stage III are most intensively invaded (L_3 – Table 5). This is understandable in view of the longer period of contact with

Table 5

Results of dissecting beet fly larvae

Year	Larval stage	Larvae					
		Total		Not invaded by parasites		Invaded by parasites	
		Number	%	Number	%	Number	%
1967	L_1	248	100	241	84.8	43	15.2
	L_2	559	100	347	62.0	212	38.0
	L_3	742	100	403	67.8	239	32.2
1968	L_1	75	100	72	96.0	3	4.0
	L_2	156	100	141	90.4	15	9.6
	L_3	170	100	151	88.9	19	11.1
1969	L_1	243	100	214	88.0	29	12.0
	L_2	824	100	591	71.7	233	28.3
	L_3	1.057	100	691	65.4	336	34.6

the parasite. The percentage of invasion of the beet fly by parasites determined by dissecting larvae is far lower than the percentage obtained by the culture method and dissection of puparia. The reason is that the best grown mature larvae were taken for culture, whereas the dissected larvae were usually younger and the period of their possible contact with the parasite was generally shorter than in the case of the larvae taken for culture.

As mentioned earlier, intensified appearance of beet flies is subject to considerable variations in different years. 1967 was a favourable year for development of the beet fly and this pest appeared in relatively large numbers. The long and warm autumn contributed to the occurrence of a fourth generation of the pest, not hitherto recorded in Poland. Weather conditions were also favourable for the delicate parasitic *Hymenoptera* and it may be assumed that the intensive invasion of beet flies by parasites (average 63.8%) was an important factor limiting the population numbers of this pest in subsequent years. In 1968 and 1969 the beet fly occurred in small numbers only, not causing serious damage and only two generations appeared each year.

The results of three years of studies indicate the important role of parasites in reduction of the pest and in regulation of its abundance in successive years.

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Nematodes as Factors Reducing the Populations of Colorado Beetle, *Leptinotarsa decemlineata* Say

By

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Studies on the role of nematode parasites in the reduction of insect pests were initiated in Poland in 1965. During the first period various insect pests were dissected to discover, which entomophagous nematodes occur in Poland and in what number. A new species, *Pristionchus uniformis* isolated from Colorado beetles was described by us. Laboratory experiments carried out with this nematode showed that it is a facultative parasite of the Colorado beetle. Larvae of *P. uniformis*, penetrating into the insects, in Petri dish cultures caused 100% mortality of Leptinotarsa decemlineata within a short time after infection, i.e. in a few hours. Our experiments were continued in glass house. Potato plants were sprayed with nematode suspensions and then L. decemlineata adults were placed on the plants. A very high mortality was obtained, varying from 36 to 90%, depending on the method of application. In field experiments, nematode suspensions were applied onto the soil and L. decemlineata adults, about to descend into the soil were introduced under the isolators. The mortality among the hibernating beetles proved to be very high, in some isolators up to 100%. Dissection of dead insects dug up from the soil revealed large numbers of *P. uniformis* at all stages of development.

In 1965 studies were undertaken on the role played by insect parasite nematodes in the reduction of plant pests. During the first period of these studies our purpose was to establish, which agricultural pests are attacked by nematodes in Poland, which species of nematodes occur in Poland under natural conditions and with what degree of abundance. The next purpose of our studies was to obtain a knowledge of the biology of these nematodes, to work out laboratory culture methods and methods for forming permanent foci of infection under field conditions.

In order to obtain data on the occurrence of nematodes we began systematic dissection of large numbers of the insects occurring in cultivated fields. The most systematic investigations were made on Colorado beetle – a serious pest occurring in all parts of Poland. Using Colorado beetle as an example it would be possible to study the character of formation of infection foci and to attempt to assess the role of nematodes in natural pests control. Dissection of Colorado beetles carried out throughout the entire growing season in different habitats revealed nematodes belonging to the following families: *Steinernematoidae*, *Mermithidae* and *Diplo-*

gasteridae. The occurrence of Steinernematidae and their role in reduction of the Colorado beetle is the current object of the studies initiated. The occurrence of *Mermithidae* in the Colorado beetle, (observed in Poland for the first time by STANUSZEK in 1966), may be only sporadic. The latter author reported that in

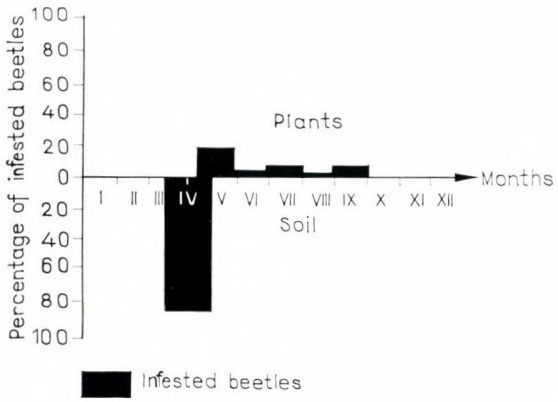

Fig. 1. Seasonal fluctuation of infestation of Colorado beetles with *Pristionchus uniformis*

some localities in South Poland foci occur in which infection may be as high as 50%, while in Central Poland the occurrence of the nematodes is vestigial. In regions where these nematodes occur in high density, they constitute an important factor in reducing the larvae and adults of *Leptinotarsa decemlineata* in summer (STANUSZEK 1970).

Results

In 1966 nematodes were isolated in our laboratory from Colorado beetles which had been dug up from the soil in early spring. This nematode, belonging to the *Diplogasteridae* family, was described by us under the name *Pristionchus uniformis* (FEDORKO and STANUSZEK, in press). This interesting species – which was discovered also in other localities – caused very high mortality, (sometimes up to 100%) in infected Colorado beetles. The occurrence of this species as parasite of the Colorado beetle varied under natural conditions, in different periods and stages of development of this pest. Colorado beetles dug up from the soil in April exhibited maximum infection and mortality, but during summer only a few individuals were infected by this nematode, and their mortality was very low. Figure 1 shows seasonal fluctuation of infection in Colorado beetles by *Pristionchus uniformis*, in fields near Warsaw.

These studies showed that it is a facultative parasite of Colorado beetle. *P. uniformis* in Petri dishes penetrated into the insects and killed them within a fairly

short period after infection. The effectiveness of these nematodes proved to be greatest within the range of temperatures from 0°C–13°C and 17°C to 25°C. In this second range rapid mortality of Colorado beetle was attained, but the nematodes did not reproduce within the insect's body and the invasive larvae of the nematode died during the course of development. With lower temperatures, on the other hand, the nematodes developed normally within the infected insects and killed them.

Greenhouse experiments were carried out using this species. Jars containing sterilized soil were placed in a greenhouse at a temperature of 22°C. Experiments

Table 1

Mortality among Colorado beetles in laboratory experiments

Variants	P. uniformis applied to		Mortality in %
	soil	plant	
I	+	–	38.6
II	+	+	90.5
III	–	+	86.4
Control	–	–	6.6

were set up in the following variants: Variant I: 1 ml of nematode suspension, containing approx. 10,000 individuals of *Pristionchus uniformis* was introduced into the soil in each of the jars, then potatoes were planted in the soil. After the plants had emerged from the soil under the isolators 10 imagines of *L. decemlineata* were placed in each of the jars. Variant II: similar to variant I except that after introducing the Colorado beetles the plants were sprayed with 1 ml of nematode suspension (concentration of approx. 10,000 individuals/ml). Variant III: potatoes were planted in to sterilized soil, then after the plants had emerged from the soil, 10 Colorado beetles were placed in each jar and sprayed with the nematode suspension.

All jars in the experiment, were inspected twice daily, and both living and dead Colorado beetles were counted. Very high mortality was obtained in different variants, depending on the method used for applying nematodes varying from 36% to 90% – whereas mortality in the control jars did not exceed 7% (Table 1).

All the dead insects were found filled with *P. uniformis* larvae, but neither development of nematodes nor production of the following invasive larvae occurred. At this temperature the nematodes acted more like a chemical and not as a biological preparation.

Other experiments were carried out by infecting the Colorado beetles during the winter diapause period, at temperatures from +10° to –16°C. Cases containing soil were sprayed with a nematode suspension (about 500,000 individuals

of *P. uniformis* per m^2). In the autumn 500 individuals of Colorado beetle were introduced per 1 m^2. In spring the Colorado beetles taken from the cases were dissected. 80% of insects were infected. In 93% of the infected insects complete development of nematodes had taken place. The infected beetles became new foci of infection.

In 1969 field experiments were set up, using a nematode suspension for infection of the soil in doses identical with the one previously mentioned, then subsequently 700 imagines of *L. decemlineata* were introduced into each of the isolators. Inspection was made before the first snow to see whether the beetles had burrowed into the soil to hibernate. It was found that within even this short time a considerable number of the beetles had died as the result of contact with the soil surface infected with nematodes. Mortality varied in different isolators from 32.7 to 40.8%, whereas in the controls the mortality did not exceed 7%. In the spring, in isolators in which nematodes had been applied, only up to 0.2% of beetles emerged from diapause, but these did not begin their feeding and died; in two of the isolators not a single insect emerged from diapause. In 81% of the dead insects dug up in the spring, the dissection revealed large numbers of *P. uniformis* in all stages of development. In the control the percentage of insects emerging from diapause was 12.6%.

Results are given in Table 2.

Table 2

Mortality of Colorado beetles in field experiments

	Initial number of insects	Number of dead individuals remaining on surface in autumn	In %	Number of living individuals in the spring	In %
Isolators with P. uniformis	2800	980	35	4	0.14
Control	2800	196	7	353	12.6

Conclusions

Our observations and experiments showed that *P. uniformis* is adapted to low temperatures. Laboratory investigations of development showed that at a temperature of 5°C the nematodes pass through the whole life cycle in 8 days. Larvae of this species exhibit activity and viability at temperatures close to zero. We consider that this is a nematode which can be successfully used for winter reduction of the Colorado beetle, especially as we have elaborated a rearing method.

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The Role of Temperature, Photoperiod and Food Quality in the Diapause of *Grapholitha funebrana* Tr. (Lep. *Tortricidae)*

By

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The results of 5-year laboratory and field experiments carried out on *Grapholitha funebrana* Tr. population in South-West Hungary (Keszthely) are summarized. G. *funebrana* swarms three times a year in South-West Hungary, i.e. it has an overwintering and two summer generations. It was observed with larvae reared in thermostate chambers at three constant temperatures (18± 1.4, 23± 1.2, 28± 0.9°C) and at different photoperiods (14/10, 15/9, 16/8 and 17/7 hours of light/dark phase) that the occurrence of diapause is determined by the photoperiod. Higher temperatures reduce in some cases the diapause induced by the photoperiod, but this effect can be equalized by the photoperiod. The critical light period lies between 14 and 15 hours; the larvae are sensible for the length of the photophase between the second and third instars. The changing temperatures (field temperatures) do not alter the critical time from the point of view of the diapause. Similarly, the number of the generation does not influence the onset of diapause. According to field observations the mass diapause of *G. funebrana* begins in the last week of July and by the 15th of August already 100% of the population is in diapause. There is a difference, however, due to different plum varieties in which the larvae had developed. The spring flight of the adults is not influenced by the circumstances to what generation of the former year the larvae belonged.

Since the marked increase of use of sterile-male (autocide) techniques, as means of biological control of insect pests, the research on the diapause has become very important all over the world. The continuous mass rearing of the species in question makes a preliminary condition to the use of any autocidal technique. The insect pests, living in the temperate climate, display a more or less extended diapause, which enables the survival of pests populations under adverse conditions. The accommodation to the unfavourable seasons became so marked in some species that the diapause could be prevented artificially only by different, simultaneously administered ecological conditions. The exact knowledge of factors inducing and terminating the diapause can be regarded, therefore, as prerequisites for developing mass production techniques.

In the light of these ideas we began our laboratory investigations in 1965, to determine the temperature (changing and constant ones), the photoperiod, the number of generations and the role of the grade of ripeness of the food in bringing about the diapause of the pest.

In contrast that in almost every country research workers dealt with *G.*

funebrana, experiments on its diapause were made until now in our institute only (SÁRINGER, 1967, 1970; SÁRINGER and DESEŐ, 1968; DESEŐ et al., 1971).

In papers discussing the development of plum moth one finds only one or two data referring to the percentage of diapause of the generations (MASHKOVICH, 1930; BOVEY, 1937; FISCHER, 1948; BŐHM, 1948; BOBIRNAC, 1958; TOSHEVA-TZVETKOVA, 1960; KOŚLIŃSKA, 1966; SEPRŐS, 1966; DESEŐ, 1967; ŞUTA, 1967).

In order to carry out exact experiments with plum fruit moth, we had to work out a method for rearing the larvae. Our method can be summarized as follows:

Every 24 hours we cutted out (together with the adjacent epidermal tissues) the eggs deposited on one plum and glued them one by one by use of flour paste on plums free of infestation. By this method we could infest hundreds of plums. The infested plums were put into glasses covered with cellophane or canvas. For pupation corrugated cardboard pieces were put between the plums. The so prepared rearing jars were placed into thermostate-chambers of different temperatures and different photoperiods. In the last five years we experimented with nearly thirty-five thousand plums. The detailed description of the method appeared in our 1967 publication.

For better comprehension of the results got in our studies we resume shortly the development of the *G. funebrana* in Hungary as follows:

G. funebrana swarms three times a year in South-West Hungary around Keszthely, which is on the geographical latitude 46 grades 46 minutes and longitude 17 grades 14 minutes, with other words, it has an overwintering and two summer generations. The swarming of the overwintering generation begins at the end of April or the first days of May.

Around the 10 and 15 of May, 50 per cent of the overwintering generation is swarming. The swarming ends in the last days of June or beginning of July. The swarming of the first summer generation begins between the 22–25 of June. That of the second generation begins on the last days of July and lasts until the end of September. In the laboratory we were able to rear a fourth generation.

The results of a great number of laboratory and field experiments and observations can be summarized, as follows:

1. At constant temperatures (in thermostate-rooms of 18, 23 and 28 centigrades) and under different photoperiods (daily light of 13, 14, 15, 16 and 17 hours a day) reared larvae showed that the diapause of *G. funebrana* is in relation with the photoperiod during the larval development. The critical light period for the induction of the diapause is between 14 and 15 hours. A day shorther than 14 hours, induces a diapause of hundred per cent. A day length between 15–16 hours prevents in great measure the development of the diapausing larvae. It has to be noticed that 17 hours light (which is in Hungary an extremely long day) the percentage of diapause stays still between 25–55. This relatively high diapause percentage shows that the species had originally only one generation and that a significant part of the population still develops in one generation.

Larvae reared at constant light and laboratory temperature stayed 57.3 per cent in diapause.

The changing daily temperature does not alter the decisive points of view of the photoperiods.

2. According to our investigations the diapause is induced in the second and third developmental stage of the larvae.

3. The high temperature (28°C) reduces in some way the diapause but its effect can be neglected as compared to the photoperiod.

4. The number of generation has not, but the grade of ripeness of the fruit has effect on the diapause at the critical time of light.

5. After our investigations, under natural conditions the mass of *G. funebrana* goes into diapause in Hungary in the last week of July. By the 15th of August there is a hundred per cent of diapause. In the going into diapause of the larvae in green, Besztercei and Olaszkék plum varieties we could notice only a small differences. The larvae developed in the greengage ripening at the beginning of August, went earlier and in greater percentage into diapause than those developed in the two other plum varieties. The late varieties ripen in the second half of September.

6. The emergence of adults in the spring was not influenced by the circumstance to which generation the larvae belonged in the previous year and in which months they went into diapause.

7. If we examine the percentage of diapause in the field and the change of the length of the day we can conclude, that the larval population developing from the last ten days of June, diapauses as a consequence of the decreasing day length. The larvae developing from June 21 on, at a 16 hour 59 minutes effective day length, receive from day to day shorter light period. This always decreasing day length causes, that the larvae developing after August 15th go in hundred per cent into diapause. The limiting day length 15 hours with regard to the critical light for the diapause, is on the 20th of August on in the region studied.

The results of investigation summarized above give important guidance to work out the method of the mass rearing in laboratory of the *G. funebrana*.

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The Effect of Temperature and Photoperiod on the Behaviour and Development of *Subcoccinella vigintiquatuorpunctata* L. (Col. *Coccinellidae)*

By

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There was a slight difference noted in the rate of development between S. vigintiquatuorpunctata individuals reared at short and long photoperiods. The greatest increase of developmental time was observed at low temperatures (generation period 40—41 days at 18°C) whereas at higher temperatures the development became faster (generation period 21—23 days at 28°C). The rate of mortality strongly correlated to temperature and photoperiod; it increased at high temperatures and long photoperiod whereas decreased at low temperatures and short photoperiod. The percentage of hiding adults was affected by different temperatures; long photoperiod and high temperatures reduced the percentage, short photoperiod and 18—23°C however increased the ratio of hiding beetles. At 18 and 23°C and at 15—16 hours photoperiod the adults of the first generation hide 38—40 days after maturation feeding and then become active again. Adults placed at 28°C and at different photoperiods (17, 16, 15, 14 and 13 hours per day) begin their feeding after maturation feeding. Results have shown that the activity of adults depends on temperature, so at high temperatures the adults remained active.

The lucerne beetle *Subcoccinella vigintiquatuorpunctata* L., is one of the commonest pests in South-western Hungary which attacks lucerne fields and causes appreciable damage. This pest does not attack only lucerne, but also about 70 other plant species with preference for lucerne and clover (JANG 1964).

In Hungary, SZELÉNYI (1944), DESEŐ (1959, 1960), and CSEHI (1964) studied the biology of this pest. CSEHI stated that it has three generations a year and the overwintered adults lay eggs in early April; the second generation appears from middle of June to middle of August and the third generation in 8th of September.

Morphological, biological, and some ecological studies were carried out by many investigators such as: GRANDI (1913), SCHMIDT (1927), MARRINER (1927), BODENHEIMER (1943), TANASIJEVIC (1958), and BONNEMAISON (1964).

BALEVSKI (1962) studied the bionomics of this pest in Bulgaria and found three generations a year. The adults of the third and in some cases those of the second generation overwinter in débris, in the upper soil level, in grass borders, or in forest edges.

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BONNEMAISON (1964) and MCMULLEN (1967) investigated the effects of temperature and photoperiod on the development and diapause of some other coccinellids.

The present experiments aimed to study the effects of different temperatures and photoperiods on the rate of development, mortality, hiding behaviour, and on the activity of adults.

Materials and Methods

Our experiments were carried out by the following methods: Adults of the lucerne beetle S. *vigintiquatuorpunctata* L. were collected with sweeping net in stands of alfalfa (*Medicago sativa* L.) at Sármellék, about 10 km from Keszthely (at the beginning of May, 1970.

The experiments were carried out in constant temperature rooms, 18°C, 23°C, and 28°C. The influence of temperature and photoperiod on the development of immature stages was determined in the thermostate chambers with photoperiods of 17/7, 15/9, and 13/11 hours. The eggs and larvae were kept in hygrostate cultures, consisting of glass-dishes (10 cm diameter and 5 cm deep) filled to its two-third with water, covered with cheese-cloth. 25 newly deposited eggs, laid by females of the stock culture were placed at 23°C in each hygrostate culture. Four hygrostate cultures with eggs were placed at every photoperiod of each thermostate room.

The use of hygrostate cultures was necessary because of the sensitivity of eggs to dry air. The hatched larvae were kept also in hygrostate cultures, in which the relative humidity was 100 per cent. The larvae were fed with top leaves of alfalfa until they completed their development. The emerged adults were kept on alfalfa plants in glass-cylinders covered with cheese-cloth. The stems of alfalfa plants were immersed into water through a perforated glass-plate (method SÁRINGER 1966). Some pieces of corrugated paper were put into the cylinders for presenting hiding places for the adults.

Results

1. Effect on rate of development and mortality

Slight differences in rate of development were observed between treatment of long photoperiod (17 hrs. per day) compared with intermediate photoperiod (15 hrs.) and short photoperiod (13 hrs. per day). The greatest difference in rate of development was due to the effect of temperature. The greatest increase of development was observed at high temperatures 28°C in which the average of generation period was ranging from 21 to 22.5 $\pm$ 1.5 days compared with 39.75 to 41 $\pm$ 1.75 days at low temperature (18°C). At optimum temperature 23°C, the mean of

generation period fluctuated from 23.5 to 27.75 ± 4.25 days. On the other hand, at identical photoperiods, high temperatures increased the speed of development and therefore immature stages developed fast, while low temperatures decreased it and immature stages needed a long period to complete their development.

Data also show that mortality increased with the increasing temperature and decreased at low temperatures.

At photoperiod 17 hrs. per day, the rate of mortality was 48% at 28°C, while it decreased to 13.5% at 18°C. At equivalent temperatures, with long photoperiod the rate of mortality, increased, while at intermediate and short photoperiods it was considerably lower.

Table 1

Influence of temperature and photoperiod on the rate of development and mortality of *S. vigintiquatuorpunctata L.*

Temp. °C	Photo-period hrs./day	No. of eggs	Mean of incu-bation period	No. of lar-vae tested	Mean of duration of different instars					Mean of generation period days	Mortality %
					1st	2nd	3rd	4th	Pupa		
18	17	25	7	96	2.5	7.5	3.3	11.5	8	39.75	13.5
18	15	25	7	91	3.0	3.0	6.0	13.0	8	40.00	12.1
18	13	25	7	97	3.0	3.0	7.0	14.0	7	41.00	7.0
23	17	25	3	90	5.0	4.0	3.0	6.0	6	27.75	29.0
23	15	25	3	95	5.5	4.0	3.0	5.5	2.5	23.50	14.7
23	13	25	4	93	4.0	4.0	3.0	6.0	6.0	27.00	16.1
28	17	25	4.5	92	5.5	3.0	3.3	6.0	2	22.30	48.0
28	15	25	5	80	3.0	3.0	3.0	6.0	2	22.00	40.0
28	13	25	6	81	2.0	3.0	3.0	4.0	3	21.00	22.3

2. Effect on hiding behaviour

We should like to mention here that the term "hiding adults" means – according to ANKERSMIT (1964) – the number of adults which finished their feeding maturity and went into diapause. The effect of temperature and photoperiod on hiding behaviour was determined by counting the number of beetles on alfalfa plants after 10 to 12 days from their emergence (MCMULLEN, 1967), and compared with those which hid inside the corrugated papers or underneath the cheese-cloth cover.

The beetles became active and began to feed 6 – 10 hours after their emergence. During maturity feeding no copulation or egg-laying was observed. The adults which had finished their feeding began to hide and went into diapause. This state will be called, summer diapause or aestivation according to ANDERWARTHA (1954). The percentages of diapausing adults are given in Table 2.

Table 2

The percentages of diapausing adults of *Subcoccinella vigintiquatuorpunctata* L. under different treatments of temperature and photoperiod

Temperature °C	Percentages of diapausing beetles	
	Photoperiods	
	17 hrs/day	13 hrs/day
18	81 + + (58)	85 (55)
23	76 (56)	97 (91)
28	37.2 (41)	52 (52)

The numbers in parentheses indicate the numbers of adults in each treatment.

As shown in table 2, at both long and short photoperiods, the increase of temperature reduced the percentages of diapausing beetles, so from 81% to 37.2% at long photoperiod (17 hours per day) and from 85% to 52% at short photoperiod (13 hours per day). We can conclude also that photoperiod had a slight effect on the percentage numbers of beetles which entered diapause. At equivalent temperatures, the short photoperiod increased the percentage of diapausing adults. These results indicate that temperature is more important in determining the adult diapause, of *Subcoccinella vigintiquatuorpunctata* L., than the photoperiod.

3. Effect on the activity of adults

At 18°C and 16-hr, and 15-hr photoperiod per day, the adults resumed their activity after 11 days and laid 22 and 87 eggs respectively.

At 23°C and 16, 15-hr photoperiod per day, adults also became active after 9 days and laid a few eggs. Eggs which were laid by those females were sporadic and the hatching ratio was very low. The low number of eggs is due to the percentage of nondiapausing beetles.

At the previous temperatures and photoperiods, adults of the first generation finished their diapause after 38–40 days and laid a huge number of viable eggs. At high temperature (28°C) the adults could not resume their activity.

Under field conditions and inside the cages which were prepared for studying the behaviour of this pest, it was observed that adults of the first generation, which emerged at the end of May, finished their maturity feeding after 24 days, then hid and went into summer diapause or aestivation for 26 days. At the end of July they resumed their activity and laid 177 eggs till the middle of August.

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On Differences in the Seasonal Activity of Cereal Bugs and Notes on the Specific Composition of their Populations in Hungary

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In the Hungarian cereal fields several bug species occur, but two *Eurygaster* species *(E. austriaca, E. maura)* and two *Aelia* species *(A. acuminata, A. rostrata)* are more common. In the seasonal activity of these pests substantial differences have been established. These differences seem to be constant, as in several localities and in consecutive years of different weather conditions, identical tendencies have been observed. The composition of the populations is decidedly influenced by several factors. It is certain, however, that due to unfavourable weather the proportion and density of *E. austriaca* and *A. rostrata* drops more rapidly within the populations than that of *E. maura* and *A. acuminata*. At the same time, under favourable weather conditions the rate of increase in the population density of *E. austriaca* and *A. rostrata* is greater than in the populations of *E. maura* and *A. acuminata*. Previous statements concerning the population dynamics of *E. austriaca* and *E. maura* seem to be valid also for *A. rostrata* and *A. acuminata*. The harvest on cereal fields seems to have a much greater influence on the populations of *E. maura* and *A. acuminata* than on *E. austriaca* populations. In most of the fields their populations have to be destroyed in a much greater proportion than the ones of *E. austriaca* and *A. rostrata*.

In Hungary and in the neighbouring countries several bug species can be found in cereal fields (MANNINGER and MANNINGER, 1933, BULLMANN and FABER, 1958.). Their seasonal activity was considered to be similar, though, MANNINGER and MANNINGER (1933) indicated long ago that *Eurygaster austriaca* appears in the cereal fields earlier than *E. maura*. In an earlier paper (BENEDEK, 1971) it has been pointed out that there exist substantial defferences in the seasonal activity of certain species. In possession of more recent data, however, there is a possibility for clearing up further details and supplementing the previous findings.

Materials and Methods

The proportion of species has been established on hand of the material collected on more than 300 localities in the 19 counties of Hungary both in 1969 and 1970. In one cereal field of each locality 200 net-sweeps were made. The characteristics discussed below can be evaluated based on ample specimens only, wherefore, cases in which less than 200 bugs had been collected in a county in one of the

years, were neglected. Altogether more than 30000 specimens have been examined. The results are shown in Table 1.

Examination of the seasonal activity of the species has been based on the material of the Hungarian Natural History Museum and on that of other institutes as well as the data published in recent faunistical literature (HALÁSZFY, 1955, BENEDEK, 1967). The data have been evaluated by means of the museum method (SOÓS, 1958): the specimens collected on a specific spot on a specific date represent-

Table 1

Specific composition of cereal bug populations in different counties of Hungary in 1969 and 1970

Year	County	Surveying period	Proportion of species, %				Number of specimens examined	Number of fields examined
			E. austriaca	E. maura	A. acuminata	A. rostrata		
1969	Szabolcs	2—17. VII	65.4	34.2	0.4	+	2584	42
	Borsod	12—18. VII	61.9	35.1	3.0	—	1839	16
	Heves	21—25. VII	46.4	43.7	7.7	2.2	441	10
	Baranya	30. VI—8. VII	45.6	49.7	4.5	0.2	581	10
	Pest	3—16. VII	44.9	51.2	3.2	0.7	1357	35
	Somogy	27. VI—16. VII	40.1	57.2	2.7	—	1531	17
	Győr	2.—14. VII	39.8	56.2	4.0	—	399	10
	Hajdú	26. VI—18. VII	39.4	57.1	2.1	1.4	4076	40
	Csongrád	4—9. VII	38.2	61.0	0.8	—	630	6
	Bács	2—4. VII	37.8	60.4	0.9	0.9	1481	28
	Békés	7—14. VII	37.6	62.4	—	—	1017	14
	Nógrád	2—10. VII	37.1	53.2	9.3	0.4	967	20
	Tolna	2—21. VII	34.9	61.0	4.1	—	1154	7
	Szolnok	3—7. VII	33.8	65.6	0.6	—	954	18
	Fejér	8—15. VII	28.7	68.5	2.8	+	3146	10
	Komárom	9—21. VII	24.3	73.4	2.3	—	432	6
1970	Pest	23. VI—9. VIII	55.5	43.5	0.8	0.2	628	47
	Szabolcs	11. VII—25. VII	38.2	57.4	4.0	0.4	526	28
	Somogy	11—25. VII	37.1	47.4	15.2	0.3	1243	21
	Csongrád	24. VI—9. VII	34.5	62.5	0.3	—	518	24
	Bács	6—16. VII	33.3	64.7	1.7	0.3	818	19
	Baranya	2—9. VII	31.3	68.4	0.5	—	798	18
	Borsod	8. VII—4. VIII	27.6	60.9	10.3	1.2	246	22
	Szolnok	8—15. VII	26.9	61.9	10.8	0.4	1199	16
	Heves	20. VII—6. VIII	22.4	64.5	13.1	—	513	24
	Fejér	6. —22.VII	22.0	87.0	0.7	0.3	1238	22
	Komárom	2—9. VII	11.2	88.6	0.2	—	297	10

Note: + indicates values less than 0.1%

ed a single data. The data are grouped according to the date of the collecting and are shown in Figs 1 – 2. In our case the grouping into decades, viz.: dissecting the months into 10 day spells was the most expedient. The material of nearly 100 years collectings', that is, the data of more than 3700 bugs have been evaluated and, therefore, the main shortcomings of the museum method are eliminated (cf. BENEDEK and JÁSZAI, 1971). In discussing and/or interpreting the results the limits of the method (BENEDEK and JÁSZAI, 1971) are being taken into account.

In 1970, in one and the same cereal field on almost 20 spots of the country bugs were collected by 200 net-sweeps weekly up to the time of the harvesting. The weather conditions of the year, however, were unfavourable for the cereal bugs, with the result that rather limited amount of data-complexes could be obtained in which the various larval or nymphal stages and adults of both of the two most important bug species were represented to a significant proportion. Based on the suitable data-complexes the percentage proportion of different phases has been established concerning both *Eurygaster austriaca* and *E. maura* respectively (Table 2).

Table 2

Percentage proportion of different stages in populations of Eurygaster austriaca and E. maura resp. in 9 spots at different dates (1970)

Locality	Date	E. austriaca				E. maura			
		adult	L_5	L_4	L_3	adult	L_5	L_4	L_3
Keresztespuszta	2. VII	25	75	—	—	—	42	58	—
	9. VII	62	38	—	—	31	65	4	—
Kunszentmárton	2. VII	11	45	33	11	—	40	60	—
	10. VII	57	41	2	—	4	71	23	2
Tata	2. VII	—	21	70	9	5	5	65	25
	22. VII	78	22	—	—	32	55	13	—
Deszk	4. VII	54	37	9	—	—	77	16	7
	9. VII	84	16	—	—	19	68	13	—
Toponár	4. VII	1	56	29	14	1	3	73	23
	11. VII	18	80	2	—	2	58	38	2
Tényő	9. VII	45	65	—	—	11	70	16	3
	23. VII	83	17	—	—	76	17	7	—
Kocs	2. VII	—	27	50	23	6	3	70	21
	8. VII	16	36	48	—	6	48	43	3
	22. VII	73	27	—	—	62	34	4	—
Hajdúnánás	15. VII	27	72	1	—	3	72	25	—
	23. VII	76	24	—	—	72	20	8	—
	6. VIII	100	—	—	—	96	4	—	—
Gyöngyöstarján	17. VII	60	40	—	—	16	64	20	—
	24. VII	70	30	—	—	20	63	17	—
	28. VII	79	21	—	—	38	62	—	—
	1. VIII	75	25	—	—	91	9	—	—

Finally, based on the material obtained in the course of the widespread surveys in the preharvesting period in 1969 and 1970 the proportion of larvae and adults both of *Eurygaster austriaca* and *E. maura* populations respectively has been established related to counties (Figs 3–4).

Results

Specific composition of cereal bug populations

In the material of the surveyes in the preharvesting periods the following bug species were represented: *Eurygaster austriaca* (SCHRANK, 1778), *E. maura* (LINNAEUS, 1758), *Aelia acuminata* (LINNAEUS, 1758), *A. rostrata* (BOHEMAN, 1852), *Dolycoris baccarum* (LINNAEUS, 1758), *Lygus rugulipennis* (POPPIUS, 1911), *Carpocoris fuscispinus* (BOHEMAN, 1849), *C. purpureipennis* (DE GEER, 1773), *Holcostethus vernalis* (WOLFF, 1804), *Psacasta exanthematica* (SCOPOLI, 1763), *Eurydema ornatum* (LINNAEUS, 1758), *E. oleraceum* (LINNAEUS, 1758), *Eusarcoris aeneus* (SCOPOLI, 1763) as well as several predatory species. Out of the phytophagous bugs, however, only the first four ones occurred in masses, while the proportion of the remaining taxa was 0.1% or even less. The majority of these bugs do not feed on gramineae, therefore, neither on cereals, but their food plants grew as weeds in the cereal fields – like cruciferous weeds for *Eurydema* species and compositae for *Carpocoris* specimens. Among the more than 30000 bugs examined not a single *Eurygaster testudinaria* (GEOFFROY, 1785) – registered as cereal bug by some authors – was found. This species lives – according to my observations – only on wild grasses and occurs mainly in clearings of forests in mid-mountainous regions.

Consequently, there are two *Eurygaster (austriaca, maura)* and two *Aelia (acuminata, rostrata)* species damaging in cereal fields but the proportion of these species varies considerably per year and by regions (Table 1). The majority of the populations is made-up by *Eurygaster* species, the proportion of *Aelia* species is seldom over 10%, thus, the role of *Eurygaster austriaca* and *E. maura* is decisive. It seems that among the *Aelia* species *A. acuminata* is larger in number, while the role of *A. rostrata* is rather insignificant. The varying proportion of the species may be traced back to the differences in the ecological conditions of the given areas as well as to the differences in the inclination to migration relating to different species (cf. BROWN, 1965). The proportion of species, therefore, is related to the location of hibernating places suitable for the species decidedly inclined to migrate, in other words, related to the location of the mountains.

The proportion of the species is changing year by year which is in function with their population dynamics (BENEDEK, 1971). It is conspicuous that in 1970, when the density of bugs was much smaller due to the unfavourable weather than in the previous year, the proportion of *Eurygaster maura*, was singificantly greater, while that of *E. austriaca* was decidedly smaller than in 1969. The changes in the proportion of *Aelia acuminata* and *A. rostrata* respectively points to parallel ten-

dency (Table 1). It seems that under unfavourable weather conditions the drop in the density of *Eurygaster austriaca* and *Aelia rostrata* populations respectively is much greater than that of *E. maura* and *A. acuminata* respectively.

Evaluation of activity diagrams based on collection data

In springs of favourable weather conditions the cereal bugs may leave their hibernating places very early but according to the data under review they occur in masses by end of April or early in May (Figs 1–2). While the earliest data of *Eurygaster maura* (Fig. 1a) precede those of *E. austriaca* (Fig. 1b), the latter seems to be much more frequent in the early period than *E. maura* because the number of early data compared with the total number of data is several times greater than in the case of *E. maura*.

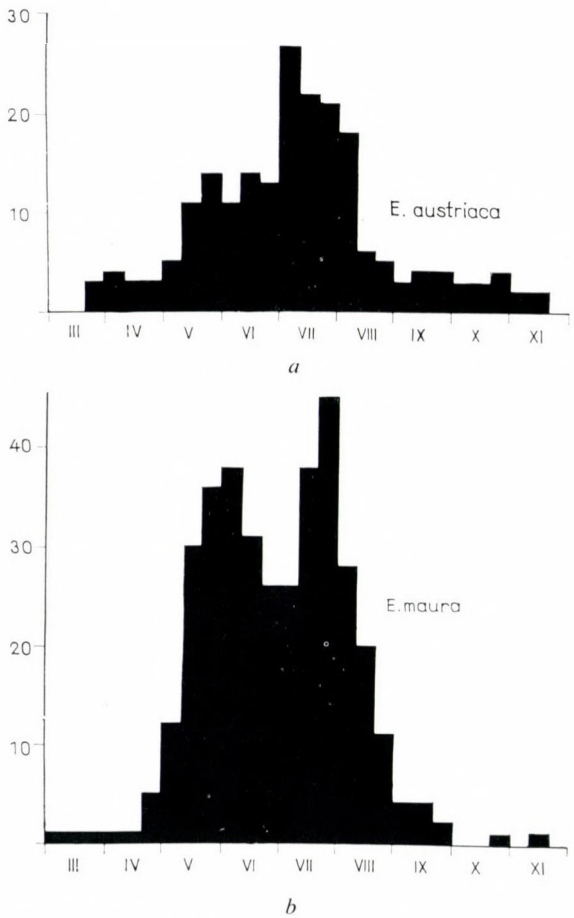

Fig. 1. Seasonal activity of *Eurygaster austriaca* (a) and *E. maura* (b). Scale: number of data

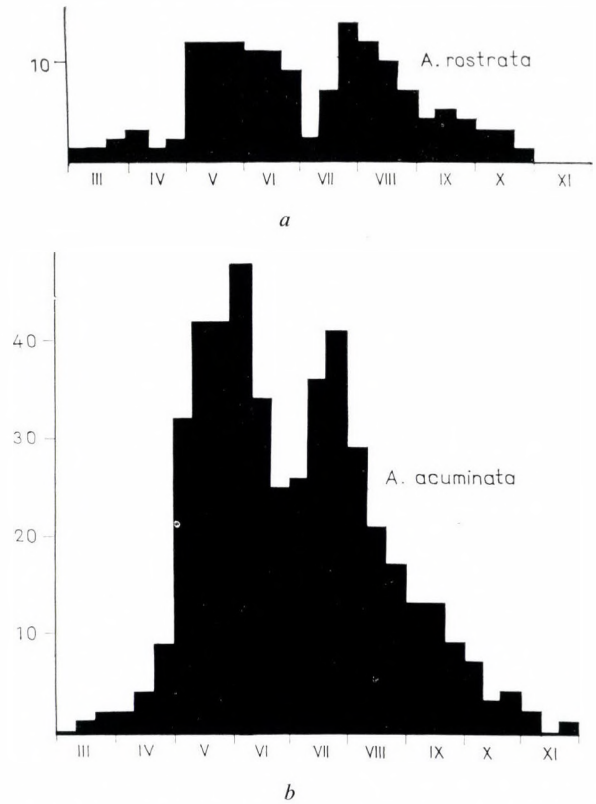

Fig. 2. Seasonal activity of *Aelia rostrata* (a) and *A. acuminata* (b)

Having left their hibernating places in the spring the activity of cereal bugs is gradually increasing and the number of hibernated populations culminates by end of May or in the first part of June. Thereafter, the number of hibernated bugs is reducing and the reduction becomes significant by end of June and beginning of July respectively. In the case of *Eurygaster austriaca*, however, there is no reduction in the population-density and in the case of this species the culmination of the new generation takes place in a much earlier period (Fig. 1a). This indicates that in the case of this species the new generation is developing definitely sooner which means that the mass occurrence of the new adults coincides with the decrease in the density of the hibernated populations. The second peak appears in the second half of or by the end of July, in the case of *E. maura* and *Aelia* species (Figs 1–2), wile with *E. austriaca* that takes place decidedly round about the first part of July (Fig. 1a).

The population-level drops rapidly after the culmination of the new generation. In the case of *Eurygaster maura* and *Aelia* species the drop is rather gradual

in August, while with *E. austriaca* it is very abrupt. This fact tells of the earlier emigration and earlier retirement into hibernation of this species. In the autumn there appears a difference as well in the activity of *E. maura* and *Aelia* species respectively. As seen in the activity diagram of *E. maura* (Fig. 1b) the drop in the density took place noticeably earlier and more rapidly than in the case of *Aelia* species (Fig. 2). This proves that *E. maura* emigrates from the cereal fields and looks for hibernating places sooner than *Aelia* species.

Results of field surveys

The quantity of *Eurygaster austriaca* and *E. maura* in the field material obtained weekly has considerably surpassed that of *Aelia* species, therefore, the former species only are being dealt with below. Results (Table 2) indicate that in the case of *Eurygaster austriaca* the proportion of more developed larvae and/or adults is always greater than with *E. maura*. The scale of differences varies by sites but same is always noticeable and in most cases considerable. The differences drop towards the latest periods (22 July – 6 August) only while in earlier periods same is very conspicuous (Table 2). The proportion of adults in the *E. austriaca* populations was – in the first part of July – the multiple of that in the case of *E. maura*. This fact decisively influences the fate of te populations, since, harvest takes place in the first part of July on most of the cereal fields.

The results of surveys in preharvesting period in 1969 and 1970 reflect tendencies similar to those mentioned above (Figs 3 – 4), i.e. the proportion of adult in the *E. austriaca* populations has everywhere been considerably greater – usually at least twofold – than in *E. maura* populations. These results direct attention to the great stability of the phenological differences, since, the tendency is manifested

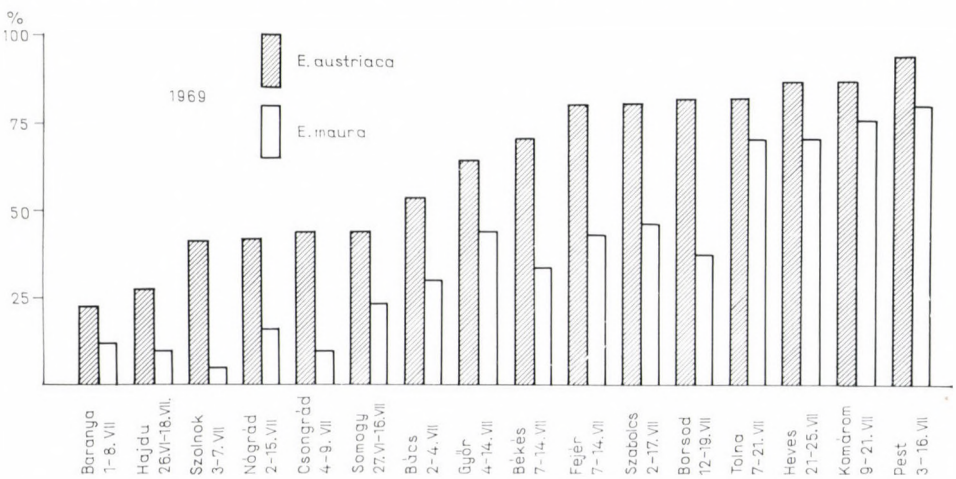

Fig. 3. Percentage proportion of adults in the populations of *Eurygaster austriaca* and *E. maura*, in the preharvesting period of 1969. Below: counties and surveying periods

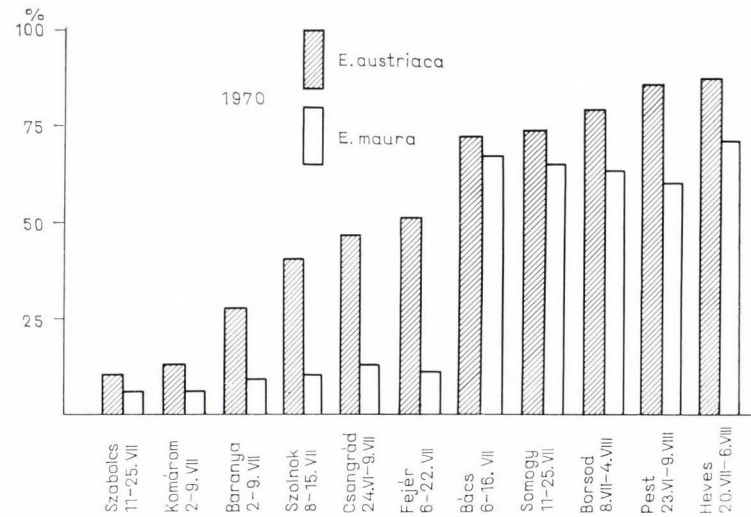

Fig. 4. Percentage proportion of adults in the populations of *Eurygaster austriaca* and *E. maura*, in the preharvesting period of 1970

very decidedly in all the counties surveyed. Weather conditions in 1969 were rather favourable for the bugs, while a remarkably cool and rainy summer in 1970 proved to be unfavourable for their development. The similar tendency in phenological differences, therefore, indicates that same is independent from changes in weather conditions.

From the results it may be revealed that the differences in the seasonal activity of different species are more definite in earlier periods and/or, generally, in the case of smaller adult proportions (Figs 3–4). In cases, however, when the proportion of the adults happens to be high within the *Eurygaster austriaca* populations, the differences between *E. austriaca* and *E. maura* respectively seem to decrease. On one hand, in the interim period the larvae in the *E. maura* populations gradually become adults, on the other hand, *E. austriaca* adults which have developed earlier emigrate gradually, therefore, their proportion reduces in the cereal fields. The proportion of adults in the *E. austriaca* and *E. maura* populations respectively is not harmonious in all cases with the period examined as far as the different counties are concerned. This is due to the differing climate and weather conditions of the various regions.

Discussion and Conclusions

In the Hungarian cereal fields several bug species occur but two *Eurygaster (austriaca, maura)* and two *Aelia (acuminata, rostrata)* species are multitudinous. *Eurygaster* species are always in vast majority (Table 1). The composition of the

populations, however, is decidedly influenced by several factors. It is certain, however, that due to unfavourable weather the proportion and density of *E. austriaca* and *A. rostrata* drops more rapidly within the populations than that of *E. maura* and *A. acuminata* (Table 1). This, at the same time, indicates that under favourable conditions the rate of increase in the population density of *E. austriaca* and *A. rostrata* is greater than in the populations of *E. maura* and *A. acuminata* respectively. Previous findings concerning the population dynamics of *E. austriaca* and *E. maura* respectively (BENEDEK, 1971), therefore, seem to be valid concerning *A. rostrata* and *A. acuminata* as well. The drop of larger-scale in the density of *E. austriaca* populations proves that due to unfavourable weather or in unfavourable periods its population density will be much lower than that of *E. maura* (BENEDEK, 1971). At the same time it seems to be sure that a similar tendency exists in the case of *A. rostrata* as against *A. acuminata.*

In the seasonal activity of the species under review substancial differences have been established. As known from earlier data, *Eurygaster austriaca* appears much earlier in the cereal fields than *E. maura* and *Aelia* species (MANNINGER and MANNINGER, 1933). This statement has been substantiated by our results (Figs 1 – 2) and field observations. The seasonal activity of *E. austriaca*, however, is substantially deviating from that of the remaining cereal bug species. No doubt, *E. austriaca* begins egg-laying much earlier, wherefore, its larvae appear in a decidedly earlier period. For this reason, in a certain period the proportion of developed larvae and/or adults in *E. austriaca* populations is always greater than in the populations of *E. maura.* This difference is constant, since, in several places and/or counties, as a matter of fact, in consecutive years of different weather conditions identical tendency has been observed (Table 2, Figs 3 – 4). The differences in the composition of the populations of the two species begin decreasing in a later period because by the second half of July the *E. maura* larvae are moulting into adult stage in ever increasing quantities and the emigration in the *E. austriaca* populations begins and continues more and more intensively. The differences in the proportion of larvae and adults respectively are especially distinct and of large extent in the first part of July which condition considerably influences the fate of the populations.

Earlier retirement into hibernation in the case of *E. austriaca* is suggested by the results (Fig. 1a), as a matter of fact, similar tendency was observed in the course of field observations as well. The decreasing in the activity of *Aelia* species in the autumn is still later and less intensive than in the case of *E. maura* (Figs 1a – 2). These species, therefore, leave the cereal fields still later than *E. maura.* In the course of field observations similar phenomena have been observed.

On the greatest part of the cereal fields harvest takes places in the first part of July. In this period the proportion of adults in *E. austriaca* populations is much greater than in *E. maura* populations. During the harvest the adults only are able to leave the cereal fields and/or fly away from the grains discharged by the combine. The harvest, therefore, has a much greater influence on the populations of *E. maura*

than on *E. austriaca* populations. The fate of the populations of *Aelia* species but mainly of *A. acuminata* is similar or still more unfavourable than in the case of *E. maura*. In fields harvested early, the *E. austriaca* populations too, are partly destroyed while in those harvested later *E. maura* too, can develop undisturbed in a significant extent. In most of the fields, however, *E. maura* populations and those of *Aelia acuminata* are destroyed at least in a twofold proportion than the populations of *E. austriaca* and *A. rostrata* respectively.

Phenological differences are very important in respect of the population dynamics of cereal bugs. Their relations concerning the population dynamics and the origin of the cereal bugs in Hungary have been discussed in a previous study (BENEDEK, 1971).

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In the preharvesting periods of 1969 and 1970 as well as in the course of the weekly examinations in 1970 the surveys were made by the District Plant Protection Stations. The collected material has been identified — under the author's guidance — by Mr. J. NOVÁK in 1969 and by Mr. K. BIBER in 1970 (District Plant Protection Station, Nagytétény). The author expresses his sincere thanks to them and to all those who participated in the surveys. The author is indebted to Dr. Á. SOÓS (Hungarian Natural History Museum, Budapest) for making available for studying the collection material of the Natural History Museum. Thanks are also due to Mrs. GY. MOHAI, Mr. J. SURJÁN, Mr. F. NAGY (Forecasting Centre in the Central Laboratory of the Hungarian Plant Protection Service, Budapest) for their assistance in evaluating the data and their instructive criticism on the manuscript. I wish to take this opportunity of thanking Director Dr. T. JERMY and Dr. L. SZALAY-MARZSÓ (Plant Protection Research Institute, Budapest) for their interest and encouragement. Dr. L. SZALAY-MARZSÓ has checked the English text for which I also express my sincere thanks.

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New Approaches to Pest Control

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Juvenile Hormone Analogues and their Theoretical and Practical Significance

By

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The analogues of juvenile hormone are substances of natural and synthetic origin, which in line with the hormone of corpora allata arrest postembryonic morphogenesis and thus make impossible the reproduction of insects. In the embryonic period their action results in the mechanical inability to hatch from the eggs. The mechanism of their action lies in the renewal of the growth ability of those parts of the body (the ability of replication of the DNA molecules) which had lost this property in the course of morphogenesis. At present over 500 compounds are known of this type, all very efficient. They can be divided into 8 groups according to their chemical structure. Their great theoretical and future practical significance is explained in the present paper and the preconditions for their utilization in the pest control are outlined.

One of the most important discoveries of entomology in the past 50 years was the discovery of insect hormones. In addition to their basic theoretical significance their perspective value as a tool of an entirely new type of insecticides is growing more and more apparent. The most important position in this respect is no doubt attributed to the analogues of the juvenile hormone. Thanks to the intensive research of chemists and biologists during the past five years we know at present more than 500 chemical substances, having most varied chemical structure, which, to a greater or lesser extent, however, being of character of biocatalyzers, show morphogenetic effects corresponding to the hormone of the corpora allata.

Their immense advantage from the practical viewpoint of insect control is, apart from their very high effectiveness which is hundredfold superior to the most effective insecticides of the DDT type and organophosphates hitherto known, the specificity of action of many of them. This conditions their harmlessness not only to man and higher animals, but frequently also to other groups of insects. Moreover, it is not a matter of toxic effect, even in the case of insects on which the analogues have an action — in reality, it is a physiological effect on morphogenesis which, in its consequences, is destructive to the insect. Their greatest promise lies in the fact that they enable to attack individual pests without endangering at the same time their entomophagous parasites. The disadvantage of hitherto applied chemical insecticides has been that in view of the smaller size of entomophagous insects these are usually destroyed much more thoroughly than the actual pest, so

that in a short time after completion of the chemical campaign we are threatened with a new, even worse calamity. The use of these insecticides in agriculture has been therefore justly compared with the use of narcotics by man – once they are applied, we are forced to use them permanently and at a growing rate in view of increasing resistance. On the other hand, the use of the analogues of JH which do not destroy the entomophagous parasites results in that these concentrate on the remaining specimens of the pest which escaped our treatment and complete thus the job for us. In this depends a real promise of eradicating the insect pests.

If we wish to grasp the principle of action of the JH analogues (aJH), we must briefly remember the notions on the way of action of the juvenile hormone and of insect hormones generally. The juvenile hormone (JH) is produced by a specific, usually paired, endocrine gland, the corpora allata, and is one of the three most important insect hormones usually designated as metamorphosis hormones. In the first place it is a so-called activation hormone (AH), a typical neurohormone originating in the neurosecretory cells of the middle part of the protocerebrum (pars intercerebralis) in the insect brain. This hormone acts as an activator of most of the metabolic processes in the insect body, primarily of secretion, including the secretion of prothoracic glands, the source of the second of the metamorphosis hormones, the moulting hormone. AH deficiency is the primary cause of insect diapause.

The second metamorphosis hormone is, as said before, the product of the prothoracic glands or of analogic organs (ventral head glands, peritracheal glands), the moulting hormone (MH) or ecdyson, the first of the insect hormones and invertebrate hormones in general, which has been identified chemically (a steroid of the keton character with five OH groups) and recently even synthetized. It acts primarily on the secretory activity of epidermal cells and of the other tissue of ectodermal origin thus inducing the moulting process. In consequence a number of other processes in the insect organism becomes secondarily dependent on it, in particular morphogenesis and to a certain extent also growth.

The true morphogenetic hormone that intervenes into the very essence of morphogenesis, however, is solely the juvenile hormone or neotenin, the hormone of the corpora allata. Its effect corresponds in many ways with the effect of somatotropine from the adenohypophysis of vertebrates. Its abundance produces giant supernumerary larvae, its deficiency, in contrast, miniature premature adults. Thus it supports growth but prevents morphogenesis not only in the postembryonal period but, as has been recently shown by means of aJH, also in the stage of embryonic development (NOVÁK, 1969). It can be proved that this effect is due to the stimulation of those parts of the body which in the absence of JH are incapable of further growth and whose gradual inactivation in the course of ontogenetic development is the immediate cause of morphogenesis. The action of JH conditions the proportionate growth in the larval period and hence it is the cause of the origin of metamorphosis in phylogenesis. It has been shown that metamorphosis is essentially a delay of the latest phase of embryonic development to the

end of the postembryonic period (NOVÁK, 1966). Related with this basic effect are a number of secondary effects, so that JH intervenes practically into all significant processes in the insect organism. Recently the hypothesis has been advanced that the essence of JH action lies in the renewal of the replication ability of those DNA molecules which had lost it in the course of morphogenesis (NOVÁK, 1966b, 1971)

One of the reasons supporting the mentioned hypothesis is the fact that entirely in conformity with JH is the action of its mentioned more than 500 analogues being of a very diverse chemical composition. The only difference that has been proved so far between them and the endogenous JH is the specificity of their effect which, however, has probably nothing in common with the principle of their action. If so many diverse substances act in an identical manner, then the mechanism of their action must be very general. With this corresponds the general occurrence and almost omnipresence of nucleic acids in living matter.

The history of the discovery of substances of this type is very short and its rapidly growing pace is typical for the development of science as a whole as well as for the majority of its most important disciplines so as for the development of individual important discoveries. A sort of prelude was the finding by WILLIAMS (1956) that the abdomen of the mature male *Hyalophora cecropia*, a giant silkworm of the family *Saturniidae*, contains an unbelievable amount of a substance (according to estimate about 2000 units) which in its effects corresponds to that of JH and which can be obtained from it by mere extraction with ethylether. At the same time ether extractions not only from related species but even from the female of the same species are virtually ineffective. The substance was considered to be the hormone of c. allata and the greater part of authors regard it as that, however, the direct evidence remains to be brought.*

Soon after, in a rapid sequence, a similar effect was discovered in a number of extracts from the most varied tissues not only of insects and other invertebrates, a.o. in cow milk and later also in the extracts from many plants and microorganisms (for review see SCHNEIDERMAN and GILBERT, 1964, and NOVÁK, 1966). SCHMIALEK (1961) in collaboration with KARLSON (1959) and WIGGLESWORTH (1961) showed that applied in the same manner a relatively simple alcohol of plant origin, i.e. farnesol and a number of its derivatives is also highly effective. Shortly afterwards SLÁMA (1963) disclosed that a number of saturated and unsaturated fatty acids, some fatty alcohols and other natural and synthetic substances produce a juvenilizing effect of various intensity.

The present ever increasing interest of physiologists, chemists and workers in plant protection for substances of this type was aroused after the discovery by SLÁMA of the so-called paper factor (SLÁMA and WILLIAMS, 1965, 1966, etc.) which was given by Prof. WILLIAMS true American propaganda which, in my belief, in view of the perspective significance of these substances, was well justified in this

* The conclusions drawn from the first verification of the effect of these substances, i.e. that it is a pathogenic effect of another type (see CARLISLE et al., 1966) has proved to be incorrect.

case. The interesting effect of every kind of paper of North American origin on the bug *Pyrrhocoris apterus* was reported at the time not only in every specialized paper, but also in newspapers all over the world. The history of this discovery is interesting, too. Karel SLÁMA, when departing for a one year study visit to Prof. C. M. WILLIAMS at Harvard University, took with him the eggs and larvae of the most popular experimental animal in our laboratory, i.e. of the bug *Pyrrhocoris apterus* for his planned experiments. He found that in America he was simply unable to breed from this bug adult insects. All the larvae developed into giant supernumerary larvae just as if he had implanted them with active corpora allata. When discussing the matter in writing I expressed the view that WILLIAMS perhaps managed to isolate the actual juvenile hormone and without telling anybody, just for the fun and for showing its effectiveness, secretely influenced SLÁMA's bugs. I had had with him before that a series of discussions on this topic. However, a detailed analysis of this phenomenon revealed something quite different. It was found that already a short contact with any kind of paper of North American origin has a similar effect on the bugs – in this case it was the filter paper put into the breeding jar – whereas paper of European origin was entirely ineffective. Further investigations showed it to be a substance contained in the wood of some widespread North American coniferous trees, e.g. balsam fir *(Abies balsamea)* used in the manufacture of paper, i.e. a terpene of the group of isoprenoids which was called juvabion after its isolation. Its effectiveness is almost unbelievable – the amount of the fraction 0.001 mg cr very short contact with the mentioned paper induces already the full effect of the juvenile hormone. At the same time this effect is so much specific that according to existing experience, apart from several other species of the *Pyrrhocoridae* family, (e.g. species of the genus *Dysdercus*) it has not the slightest effect not only in other orders of insects, but even on the bugs of the closest relative families, like the *Lygaeidae* or *Pentatomidae*.

The huge interest aroused by this discovery resulted in the fact that at present groups of chemists in a number of countries, a.o. more than 10 workers in our Institute for Organic Chemistry of Czechoslovak Academy of Sciences are studying substances of this type. As a result there exist today over 500 compounds with a full juvenilizing effect, many of them differing in their effectiveness on individual insect groups. Beside of more or less specific substances there are also substances having a general efficacy. The hitherto existing substances can be divided according to their chemical structure, into the following eight groups (cf. SLÁMA, 1971):

1. Substances of the type of the effective component of the extract from the abdomens of the male *Hyalophora cecropia* (see above) which had been recently isolated and determined by RÖLLER and his colleagues (RÖLLER et al. 1967) as methyl 10-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate. Their characteristic property is the epoxy group at $C_{10, 11}$ and the ethyl-radical at C_7 and C_{11}, whereby they have a structural affinity with farnesic acid (3,7,11-trimethyl-2,6,10 dodekatrien). RÖLLER and other authors regard the above mentioned substance as the actu-

al juvenile hormone. However, I do not see their reasons convincing. They are primarily effective in *Lepidoptera*, *Orthoptera* and *Coleoptera*.

2. Some fatty acids and alcohols (both saturated and unsaturated ones) and their derivatives (see SLÁMA, 1962) whose activity in comparison with the other aJH is relatively low.

3. Monocyclic sesquiterpenoids, to which belongs the mentioned juvabion, i.e. the effective substance of the "paper factor" (see above). They all are very efficient in the bugs of the family *Pyrrhocoridae* and virtually ineffective in other groups of insect.

4. A separate group is formed by the aromatic derivatives of juvabion the effect of which is, according to experience, equally limited to the bugs of the family *Pyrrhocoridae*.

5. Closest to the cecropia factor are acyclic terpenoids similar to the derivatives of farnesic acid but without the epoxyd group and with a methyl radical instead of ethyl-radical at C_7 and C_{11}. This group embodies the mostly studied aJH, such as dichlor-dimethyl-farnesoate. To this group compounds can be assigned in which some carbon atoms in the basic chain can be substituted with atoms of oxygen or nitrogen. They are all widely efficient.

6. The most extent group of aJH is represented by aromatic terpenoid ethers, thioethers, amines, etc. These are mostly derivatives substituted by the derivatives of phenols or anilines in the p-position, having an acyclic mono-or sesquiterpenic chain differently modified or substituted. They include a number of highly effective aJH with a close specificity of effect in various insect groups.

7. Acyclic compounds of the dodecyl ether type having a branch-free chain and other compounds with juvenile activity, such as synergists of insecticides of the sesamex type.

8. Very significant, also from the theoretical aspect, is the most recently discovered group of compounds of a peptidic structure (ZAORAL and SLÁMA, 1970). They all are derivatives composed of three or two amino acids and of a branched aliphatic residue. The compounds known so far also act specifically on the bugs of the family *Pyrrhocoridae*.

The peculiarity of all compounds of this type is that, due to the physiological nature of their effects, they act solely in certain sensitive periods of postembryonic development of the insects, that is, primarily in the beginning of the last larval instar. This limits somewhat the practical significance of their application to the insects in the postembryonic developmental stage. It might be objected also that since they do not kill the insects but merely prevent their maturation, their practical value is limited, for even juvenilized larvae take in food and hence they continue in causing damage. This disadvantage, however, is manifold counterbalanced by their effectiveness on the embryonic development of the insects so that they may be regarded in the first place as ovicides. The effect of some of them is so intensive that not only the minute amount in the above mentioned range is sufficient, but with the most effective of them such a slight quantity as transferred

from the male to the female during copulation will be enough to influence the further development of the eggs and prevent their hatching (MASNER, SLÁMA, LANDA, 1968). The effect of aJH is not toxic even in the insects where they are active, it merely has a physiological influence on further morphogenesis essentially in the same manner as its action in the postembryonic development (see NOVÁK, 1969). This is most evident in the fact that all aJH can replace the JH so far as its necessity for the activation of the ovarial follicle cells is concerned. The affection of the eggs induces a mechanical inability to leave the egg membranes and thus the destruction of all embryos at almost any stage of their development. In this lies the greatest promise for a practical utilization of the analogues of JH.

Prior to implementing their routine application in practice – in the form of aerosoles and in other ways – many theoretical and practical problems have to be solved. Thus chemists are interested in the relationship between the composition and chemical structure of the substance the knowledge of which would help us to produce an even greater variety of even more effective compounds. No less important in this respect is the connection between the composition of the given compound and the specificity of its effect on various groups of insects. Even though in this respect we are still in the initial stage, some conclusions can be drawn already. Thus SLÁMA (1971) points out the connection with the size of the molecule which has a certain optimum at which the compounds attain their maximum effectiveness. Next, it is the presence of a branched chain in the molecule whereby the most satisfactory appears to be the branching at every fourth carbon atom in the chain as it is the case in the isoprenoid substances. It also seems that substances with a double bond have a more specific effect and frequently a greater activity than hydrogenated substances without double bonds. Activity appears to depend on certain sites of the molecule, e.g. in terpene- like substances around the tertiary carbon atom in which the chain usually branches. Some substituents enhance the juvenilizing activity on these sites, for example, the atoms of halogenes (Cl), the oxygen of the epoxy or alkoxy groups, alkyl, etc., however, only in some insect groups, whereas in others the activity is even decreased.

Another very important problem is the penetration of the integument by the substance and its transport to the relevant tissue. Connected therewith is the effectiveness of the individual types of compounds, their stability in the organism, the time of their action in the organism, etc. In this context, it is interesting to note that whereas most of the compounds are more efficient in a subcutaneous injection, there are some of them that are more effective at a surface application and again others that are neither efficient using the one or other method but become highly effective when applied in the food. As said before, all these aspects are in the initial stage of investigation. A study on the action of other compounds and of the spectrum of action of each of them on individual groups of insects will bring some progress.

An investigation of their practical use in field conditions possesses equally a number of problems, as shown by the first attempts made. First of all it is the meth-

od of application, the form of aerosoles appearing to be most suitable. This gives rise to the problem of the best solvent with a possibly synergic effect. Important is the stability of the given compound applied on the plant surface, so as in other conditions of application. Equally important is the optimal time of application which will differ not only with regard to each species but also with regard to the specific conditions of the place, the given year, etc. Notwithstanding that, many of these problems are still to be solved, the first attempts of a practical application, for example, against the bug *Eurygaster integriceps* are promising.

A further study of these compounds deserves, no doubt, every attention, and efforts in this respect are increasing. This is due, among others, to the unsatisfactory experience with the major part of existing chemical insecticides, in particular their detrimental effect on the balance in nature, also from the point of view of the nature protection, and even more so their noxious effect on human health (for example, their accumulation in the human organism and the resulting carcinogenic action). For this reason the most advanced countries (England, USSR) have either forbidden or largely reduced by law their application in agriculture. Hence the prospect of replacing them with a type of compounds which would be even more effective and at the same time harmless to human health as well as to the whole of nature is of invaluable importance.

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Endocrine Control of Insect Reproduction, a Possible Basis for Insect Control

By

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Both juvenile hormones and their all-trans isomers induce the synthesis of a female specific protein essential for egg maturation. Dose-response curves have been established for these hormones. Actinomycin D administered simultaneously with the JH inhibited completely the synthesis of the specific protein. Only partial inhibition was observed when the drug was given 1 or 2 days after hormone treatment. These observations allow the tentative conclusion that JHs influence transcription of specific mRNA. Ecdysterone caused an inhibition of the JH effect. This inhibition was only partial even when given simultaneously with JH and in relatively high doses. The inhibition of egg maturation by ecdysones is thought to be effected via inhibition of essential protein synthesis.

In the great majority of insect species yolk deposition in the growing oocytes is controlled by the corpus allatum hormone whereas in a few species neurosecretion from the pars intercerebralis appears to be essential for egg maturation (cf. ENGELMANN, 1970). Yolk deposition is independent of any known endocrine gland only in most Lepidoptera and all Phasmida. While past research had established these basic facts more recent efforts have been concentrated on the mode of action of hormones, particularly the corpus allatum hormone or juvenile hormone. It is now thought that the juvenile hormone (JH) exerts its action via specific protein synthesis in the female insect. In several species it has been shown that hormone controlled synthesis of specific proteins is essential for yolk formation; generally 80 to 90% of the total egg proteins consist of these proteins. The rates of synthesis of specific and non-specific proteins as controlled by the JH have been determined in the viviparous cockroach *Leucophaea maderae* by precipitations of the labelled proteins with their homologous antibodies (ENGELMANN, 1969, 1971). The use of these techniques makes it now possible to give further details concerning the action of the JHs on reproductive processes in this species. Furthermore, results obtained after simultaneous application of JH and ecdysterone in the adult female provide grounds for speculation on the interaction of these two hormones in larval insects

Material and Methods

The cockroaches were reared at 26°C and approximately 80% relative humidity. Newly emerged females were collected daily and kept without food until allatectomy. Operations and treatments of the animals have been described previously (ENGELMANN, 1969, 1971). JH in olive oil was applied topically to head ligated animals and 20-hydroxyecdysone (Mann Research) dissolved in 10% ethanol was injected. Five days later the animals were pulse labelled, bleed, and aliquots of the sera were treated with TCA, and specific or non-specific antibodies as previously described (ENGELMANN, 1969). From the percentage of label precipitated by the antibodies the incorporation rates of label into female specific and non-specific proteins were calculated. For the designation of the juvenile hormones the trivial names used by MEYER et al. (1970) are employed here. Accordingly, the 12,14-dihomojuvenate is methyl 10,11-epoxy-7-ethyl-3,11-dimethyl-2,6,10-trans, trans, cis-2, 6-tridecadienoate; 12-homojuvenate or lower homologue is methyl 10,11-epoxy-3,7,11-trimethyl-2,6,10 trans, trans, cis-2,6-tridecadienoate; juvenate is methyl 10,11 epoxy-2,6, trans, trans-farnesate.

Results

As has been reported earlier (ENGELMANN, 1969) topical application of 1 μg of the all trans isomer of the first authentic JH (12,14-dihomojuvenate) to isolated abdomina of *Leucophaea* induces the *de novo* synthesis of a female specific protein at a rate comparable to that after implantation of 4 active corpora allata. In the present study dose-response curves for its synthesis were obtained for both naturally occurring JHs or their all trans isomers as well as for one of the lower homologues, juvenate (Fig. 1). Minor differences in potencies of hormones obtained from different laboratories were found. As is illustrated, in this system the all trans 12,14-dihomojuvenate was about 10 time more potent than the naturally occurring t,t,c-isomer whereas the t,c,t isomer was completely inactive (ENGELMANN, 1971). Similarly, the all trans isomer of the second JH (12-homojuvenate) was more potent than the t,t,c-isomer (Fig. 1) (cf. ENGELMANN, 1971). The methyl 10,11-epoxyfarnesenic acid (juvenate) was markedly less potent than the other compounds.

Since the dose-response curves are similar in shape, these results may allow the hypothesis that all active compounds tested influence the same molecular mechanism even though differences in potencies were found. All of the hormones induce the synthesis of one molecular species, the essential female specific protein. What is then the molecular mechanism of JH action? In an attempt to find an answer to this question, 2 μg of actinomycin D was applied simultaneously or at given intervals following JH treatment. Actinomycin D given at this dose is non-lethal and normal animals recover within a few days. The synthesis of the female specific protein was tested for as in the other assays on the 5th day after JH treat-

ment. As can be seen (Fig. 2), when JH and actinomycin D were given simultaneously no female specific protein was synthesized and synthesis of the non-specific proteins was markedly reduced. However, application of the drug 24 hr after hormone treatment did not completely abolish specific protein synthesis. Inhibition was very mild when applied at 48 hr after JH application and female specific protein synthesis was of a magnitude similar to that of the 2nd day after hormone treatment (ENGELMANN, 1971). These data may allow the tentative conclusion that JH influences the synthesis of a specific mRNA, and that this

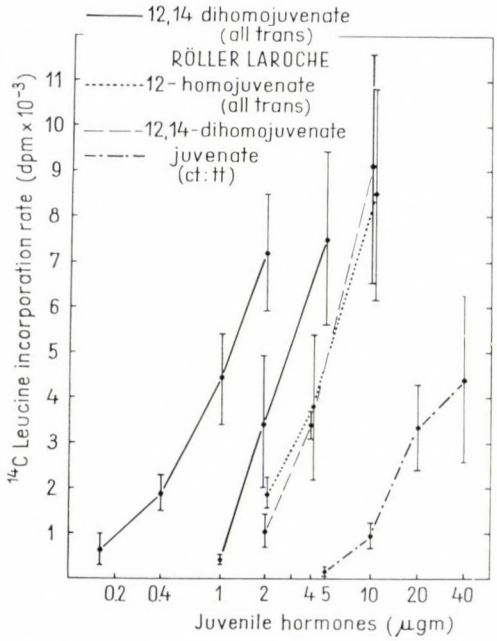

Fig. 1. Incorporation rates of ^{14}C leucine into female specific protein following treatment of allatectomized females with juvenile hormones and their stereoisomers. The all trans 12,14-dihomojuvenate was obtained from two different laboratories. Characteristic appears to be the nearly identical shape of the dose response curves. Bars are standard errors

species of messenger is relatively long lived (at least 3 days). This means that after drug application on day 2 specific protein synthesis continued at that level for an additional 3 days.

Ecdysones are known to inhibit yolk deposition in adult females of *Leucophaea* (ENGELMANN, 1959), *Musca domestica* and *Tribolium confusum* (ROBBINS, et al., 1968), and *Stomoxys calcitrans* (WRIGHT and KAPLANIS, 1970). Little could be suggested concerning the mode of action of ecdysones in these cases. The

findings could, however, lead to interesting speculations since these naturally occurring hormones may be used effectively as chemosterilants. The present study on essential protein synthesis for yolk formation may indeed provide the basis for a better understanding of the processes involved. In a series of experiments ecdysterone (20-hydroxyecdysone) was injected in given amounts. The same day the females were treated with 10 μg of the all trans isomer of 12,14-dihomojuvenate. As with the other experiments, the protein synthetic activity was assayed for

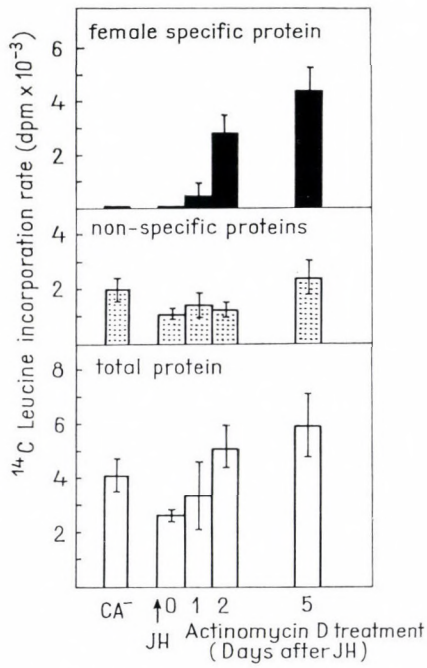

Fig. 2. Incorporation rates of ^{14}C leucine into total, female specific, and non-specific serum proteins after 1μg of the all trans 12,14-dihomojuvenate had been applied followed by actinomycin D treatment 0 to 2 days thereafter. Protein synthesis was assayed for on the 5th day after JH application in all cases. Bars are standard errors

5 days later. It was found that with increasing doses of ecdysterone the synthesis of the female specific protein decreased drastically (Fig. 3). However, even after application of the relatively high dose of 40 μg it was not completely abolished. As a consequence of a decreased specific protein synthesis the total protein synthetic activity was decreased. From this finding one may conclude that inhibition of egg maturation by ecdysone is essentially effected via inhibition of specific protein synthesis.

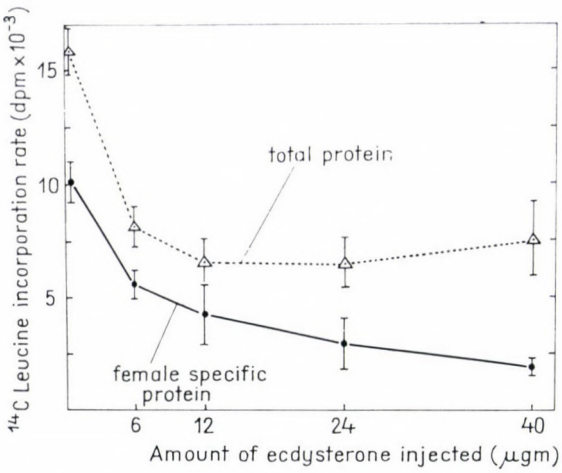

Fig. 3. Incorporation rates of ^{14}C leucine into female specific protein following the treatment of allatectomized females with 10 µg of the all trans 12,14-dihomojuvenate simultaneously with variable doses of ecdysterone (20-hydroxyecdysone). Protein synthetic activity was assayed for on the 5th day after hormone treatment. Bars are standard errors

Discussion

Immunological techniques combined with studies on endocrine deficiencies and replacement therapy lead to the conclusion that the corpus allatum hormone or juvenile hormone (JH) controls the synthesis of essential female specific proteins in *Rhodnius* (COLES, 1965), *Leucophaea* (ENGELMANN and PENNEY, 1966; ENGELMANN, 1969), *Periplaneta* (BELL, 1969), *Sarcophaga* (WILKENS, 1969), and *Leptinotarsa* (DE LOOF and DE WILDE, 1970). Unless specific proteins are produced yolk deposition does not take place in these species. Egg maturation is thus controlled by the JH via the synthesis of specific proteins. JHs are the only compounds known that cause the synthesis of the female specific protein in *Leucophaea* (ENGELMANN, 1971) as well as other species. After the antibody to the specific female protein became available, dose response curves for JH induced synthesis of the female specific antigen could be established. Both naturally occurring hormones are similarly potent, but their respective all trans isomers are somewhat more potent. However, the t,c,t isomer is completely inactive (ENGELMANN, 1971). Ethyl groups in position 7 or 11 of the JH molecule are essential for maximal activity (Fig. 1) (ENGELMANN, 1971). If all ethyl groups are replaced by methyl groups (juvenate) the activity decreases by a factor of 10 to 20. Stereoisometry and the nature of the side groups are thus important for the expression of the biological activity of the JH in the *Leucophaea* system.

Based on results obtained after actinomycin D application one may speculate that JH influences transcription of genetic information. In analogy, ecdysone is thought to act on the gene locus influencing the synthesis of specific mRNA for DOPA-decarboxylase (KARLSON, 1963). Inhibition of protein synthesis by actinomycin D is, however, no conclusive evidence for inhibition of messenger synthesis (cf. TATA, 1966) and further experiments must be performed to test this hypothesis. In this context it is of interest that ecdysterone inhibits specific protein synthesis in adult females of *Leucophaea*. Originally (ENGELMANN, 1959) it was thought that prothoracic glands or ecdysone inhibited egg maturation in this species through the inactivation of the animal's corpora allata since these glands remained relatively small after injections of ecdysone. Since nymphal corpora allata are active in the presence of active prothoracic glands it was necessary to postulate an additional nymphal factor that counteracts in part the action of ecdysone (ENGELMANN, 1959). It is now feasible to assume that ecdysone injected into the adult milieu inhibits protein synthesis, particularly the specific one, as well as the animal's corpora allata. From these results one may speculate on the fact that the female specific protein is absent in female nymphs (ENGELMANN, 1969) even though their corpora allata are active: ecdysone inhibits its synthesis. Possibly the periodic alternation of egg maturation and molting in *Thermobia domestica* (WATSON, 1964) finds now its plausible explanation, i.e. the essential female protein is not synthesized during the active phase of the prothoracic glands and therefore molting and egg maturation do not take place simultaneously.

Very little can be said about the mode of action of ecdysterone at this time. It possibly inhibits transcription of specific messenger and/or translation of it. It may compete for the site of action of the JH as the dose response curve seems to suggest. It should be mentioned that the relatively high doses of ecdysterone which had to be applied to obtain more than 50% inhibition of specific protein synthesis may be necessary because of the rapid inactivation of injected ecdysterone. Rapid elimination of injected ecdysones has been found in a variety of different insect systems.

The recent reports on inhibition of egg maturation by feeding of various ecdysones and analogues in *Musca* and *Tribolium* (ROBBINS et al., 1968) and *Stomoxys* (WRIGHT and KAPLANIS, 1970) are of interest in this context. However, in these cases no data are available on the actual amounts of ecdysones taken in during feeding. Differential exclusion or breakdown of different ecdysones or analogues by the digestive tract may account for the variable potencies of the compounds in these species. It is clear that additional work has to be done before we understand the action of ecdysones in the adult insect. Be that as it may, the finding of inhibition of egg maturation by ecdysones is of more than casual academic interest and may be of potential value for insect pest control.

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The Effective Dosis of Juvenile Hormone Analogues from the Point of View of Insect Control

By

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The author describes methods and concepts for defining the effectivity of juvenile hormone analogues in laboratory experiments, which make possible to draw up dosis-effectivity curves, compare effectivities of different treatments and study the influence of different ecological factors (e.g. temperature) on treatment effectivity. The methods described seem to be also useful for defining the duration of sensitive periods of insects from point of view of economical JH treatment and for estimating in given cases the doses to be used in the practice against insect pests.

At the present time, there are still considerable difficulties which prevent us from using juvenile hormone analogues in pest control. The greatest problem is to discover properly active and specific compounds which are applicable and unexpensive at the same time. In this situation it is reasonable that the research groups have been concentrated mostly on the biological testing of newly synthetized chemicals. Only few of these investigations has aimed at estimating activity and specifity on insect pests. Up to now, only a few field trials have been reported. Owing to the present difficulties, it seems necessary, at least in some cases, to perform laboratory experiments on various insect pests in larger scale, in order to get more detailed informations concerning the possibilities of the practical use of hormone analogues in pest control.

The aims of such investigations can be quite different. Of course, the conditions can be much more regulated and controlled in these experiments than in the practice. For example, we can predetermine the dosis of a compound to be used, or the developmental or physiological stage of an insect with desired accuracy.

In order to get useful informations for future practice, we have to consider the following requirements:

1. Treating only instars or physiological stages which can be treated in natural conditions, too.

2. Using only such treatments which have some similarities to the control methods used in the practice (e.g. topical treatments).

3. Studying only juvenile hormone effects which diminish the capability of a definite insect species to cause damage, i.e. which reduce the number of individuals, or which affect some of their vital physiological activities. So the treat-

ments should result, for example, in anatomical or physiological injuries decreasing their normal movement, feeding, or reproduction.

The present paper deals only with two aspects of the complex:

1. The *effectivity* of a juvenile hormone analogue from the viewpoint of practical use;

2. The *dosis value* being in connection with a definite JH effectivity.

On the level of laboratory screening tests, the effectivity of a JH analogue is expressed by the activity of the compound, the latter, in turn, by a dosis value (activity unit) which induces a well defined JH effect, e.g. the emergence of well-known intermediate adultoid forms in general or in 50% of the whole population treated (SLÁMA et al. (1969), SLÁMA et al. (1970), WIGGLESWORTH (1969), ROSE et al. (1968)).

In the laboratory experiments destined for preliminary steps towards future practice, the calculation of JH effectivity is influenced or rather determined, first of all, by the result which can be achieved or is to achieve on an insect species under natural conditions. Our aim can be the killing of insects; in other case, however, it is quite sufficient to reduce their feeding activity or reproduction to a required extent. The developmental injuries induced by JH treatment appear sometimes in a very complex manner (e.g. in larvae or pupae). In estimating effectivity, we must take in account all these damages, resp. the degree of the curtailment of a physiological activity in the whole population treated by JH analogue. For further investigations we have to choose a physiological activity which is vital for the insects (e.g. feeding), or is in close connection with their faculty to cause damages in the field.

JH effectivity can be expressed by a decimal fraction or in percentage. It refers, of course, to a given developmental or physiological stage, and is in close connection with a *dosis value* (expressed in μg-s per specimen, for example). An effectivity value can be taken as a basis (e.g. 0.50 or 50%), and the dosis value adequate to it can be also calculated. The latter is suggested to be named *effective juvenile hormone dosis* ($ED_{JH}50$).

The calculation of effectivity or effective dosis seems very simple in some cases. Investigating ovicidal or chemosterilant effects, the mortality of eggs, resp. the reduction of the number of fertile eggs expresses directly the JH effectivity of the compound. Treating larvae or pupae, the estimation of effectivity needs additional experiments in which the degree of decrease of the appropriate physiological activity can be established. In this latter case, calculations are more complex, in some respect.

In the followings, we want to demonstrate such a calculation procedure with reference to the evaluation of two small-scale laboratory experiments as models.

Model Experiments

a) In one experiment, young pupae of the Colorado potato beetle (*Leptinotarsa decemlineata* SAY.) of different ages were topically treated on their ventral abdominal surface with different doses of farnesyl methyl ether (FME) in a standard 1 μl acetone solution. The pupae of *Leptinotarsa* are very sensitive to isoprenoids (MELTZER and VAN HEYNSBERGEN (1965)). The pupae were kept at 23°C. After the emerging of adults, we studied the rates of developmental injuries on the different individuals treated with various doses (in different pupal ages).

Five different categories of injury could be clearly distinguished as follows:

0 – normal, uninjured adult;
I – slightly damaged imago with shortened elythrae;
II – injured adult with rudimental elythrae;
III – strongly injured adult casting off its pupal skin with considerable difficulties;
IV – insect unable to emerge.

Table 1

The effect of FME on *Leptinotarsa decemlineata* male pupae

Age	Dosis of FME in μg		No. of dead pupae	No. of living individuals	Number of individuals in categories of JH effect					Percentage				
	per spec.	per g l. w.*			0	I	II	III	IV	0	I	II	III	IV
6 hrs.	2	22	6	14	5	4	4	1	—	35	29	29	7	—
	4	44	12	8	4	2	—	2	—	50	25	—	25	—
	6	66	3	17	—	1	6	7	8	—	6	35	41	18
	8	88	5	15	1	2	—	7	5	7	13	—	47	33
	10	110	3	17	—	—	—	9	8	—	—	—	53	47
	20	220	1	19	—	—	—	2	17	—	—	—	11	89
24 hrs.	5	55	7	13	2	4	2	5	—	15	31	15	39	—
	10	110	4	16	—	1	7	7	1	—	6	44	44	6
	20	220	1	19	—	—	2	16	1	—	—	11	84	5
	30	330	7	13	—	1	4	8	—	—	8	31	61	—
	40	440	1	19	—	—	6	10	3	—	—	31	53	16
	50	550	4	16	—	—	5	9	2	—	—	31	56	13
	100	1100	9	11	—	—	—	7	4	—	—	—	64	36
48 hrs.	50	550	0	20	1	8	5	5	1	5	40	20	20	5
	100	1100	1	19	1	2	2	2	3	5	11	11	58	15
	200	2200	4	16	—	—	1	1	13	—	—	—	13	81

* live weight = 92.2 ± 2.4 mg (n = 30) ~ 90 mg

The number of individuals of an experimental group in these categories and the appropriate percentages were established. In some cases relatively high "natural" mortality was observed which could not be brought in connection with the dosis applied. The results concerning male individuals are summerized in Table 1, those concerning females in Table 2.

The feeding activity of some injured forms was studied by the leaf disc test originally developed by JERMY (1961), with some modifications. 12 small discs, cut out from potato leaves, 2 cm in diameter, were set individually at their centres on pins and put into a cup (15 cm in diameter, 6 cm high) evenly distributed around a circle about 0.5 cm above the wax-linned bottom. 5 adults were put into each cup (in 3 repetitions). The investigation was carried out at room temperature (18–22°C). After 12 hours the surface area of the potato leaf discs consumed by the insects was estimated at each experimental group, and the mean values were calculated. These mean values represent the *feeding activities* of different groups. The feeding activities of JH treated insects are expressed also in proportion to those of controls. These values indicate *relative feeding activities.*

Table 2

The effect of FME on *Leptinotarsa decemlineata* female pupae

Age	Dosis of FME in µg		No. of dead pupae	No. of living individuals	Number of individuals in categories of JH effect					Percentage in categories of JH effect				
	per spec.	per g l. w.*			0	I	II	III	IV	0	I	II	III	IV
6 hrs.	2	18	3	17	5	8	2	2	—	29	47	12	12	—
	4	36	6	14	9	2	1	2	—	65	14	7	14	—
	6	54	3	17	—	6	5	6	—	—	35	30	35	—
	8	72	6	14	1	1	3	4	5	7	7	21	29	36
	10	90	1	19	—	—	3	5	11	—	—	16	26	58
	20	180	4	16	—	—	1	3	12	—	—	6	19	75
24 hrs.	5	45	5	15	2	9	2	2	—	13	61	13	13	—
	10	90	3	17	—	6	5	5	1	—	36	29	29	6
	20	180	4	16	—	—	6	10	—	—	—	38	62	—
	30	270	3	17	—	—	3	9	5	—	—	17	53	30
	40	360	0	20	—	—	3	17	—	—	—	15	85	—
	50	450	1	19	—	—	5	11	3	—	—	26	58	16
	100	900	0	20	—	—	3	10	7	—	—	15	50	35
48 hrs.	50	450	0	20	5	7	4	4	—	25	35	20	20	—
	100	900	4	16	2	7	3	2	2	13	44	19	12	12
	200	1800	0	20	—	1	4	1	14	—	5	20	5	70

* live weight = 112.8 ± 2.3 mg (n = 30) ~ 110 mg

Table 3

Feeding activity of *Leptinotarsa decemlineata* imagos emerged from FME treated pupae

Sex	Feeding activity*			Relative feeding activity			
	control	IInd category	IIIrd category	0 and Ist category	IInd category	IIIrd category	IVth category
Male	11.4±0.6	5.3±1.5	1.3±0.6	1.00	0.46	0.11	0.00
Female	10.0±1.3	9.2±1.1	2.7±2.1	1.00	0.92	0.27	0.00

* number of potato leaf discs, 2 cm in diameter, consumed during 12 hours (n = 3)

The feeding activities, resp. relative feeding activities of the categories II and III were studied only. The individuals in category I behaved like normal insects; the animals in category IV were naturally not included in the test. The results concerning both sexes are summerized in Table 3.

In possession of these values we can calculate the (relative) feeding activity of a whole experimental group. Taking the proportional numbers (the expressions of percentages in appropriate decimal fractions) of different categories, and multiplying them one after the other by adequate (relative) feeding activities, then summarizing these values, we get the (relative) feeding activity of the whole group. (In the categories 0 and I relative feeding activity is 1.00; in the category IV it is 0.00.)

Table 4

The effectivity of pupal FME treatment on the feeding activity of *Leptinotarsa decemlineata* male imagos

Age	Dosis of FME in µg per spec.	Relative feeding activity of the whole experimental group	Effectivity of FME treatment
6 hrs.	2	0.77	0.23
	4	0.77	0.23
	6	0.27	0.73
	8	0.25	0.75
	10	0.06	0.94
	20	0.01	0.99
24 hrs.	5	0.57	0.43
	10	0.31	0.69
	20	0.14	0.86
	30	0.29	0.71
	40	0.20	0.80
	50	0.20	0.80
	100	0.07	0.93
48 hrs.	50	0.56	0.44
	100	0.27	0.73
	200	0.04	0.96

Table 5

The effectivity of pupal FME treatment on the feeding activity of *Leptinotarsa decemlineata* female imagos

Age	Dosis of FME in μg per spec.	Relative feeding activity of the whole experimental group	Effectivity of FME treatment
6 hrs	2	0.91	0.09
	4	0.89	0.11
	6	0.72	0.28
	8	0.41	0.59
	10	0.22	0.78
	20	0.10	0.90
24 hrs	5	0.90	0.10
	10	0.71	0.29
	20	0.52	0.48
	30	0.30	0.70
	40	0.37	0.63
	50	0.40	0.60
	100	0.28	0.72
48 hrs	50	0.83	0.17
	100	0.77	0.23
	200	0.24	0.76

In connection with the feeding activity of adults, the effectivity of pupal JH treatment can be demonstrated by the degree of reduction of feeding activity experimentally induced. Therefore, subtracting the value of (relative) feeding activity in a definite experimental group from 1.00 we are able to calculate a value which expresses the *effectivity of hormone treatment* in this population. The results concerning males are summarized in Table 4, the ones concerning females in Table 5.

Taking 0.50 effectivity as a basis, the *effective doses* ($ED_{JH}50$) of farnesyl methyl ether for the pupae of the Colorado potato beetle, in connection with the feeding activity of adults, fall between the following dosis values (in μg-s per specimen):

	males	females
	μg	μg
at 6 hrs	4 – 6	6 – 8
at 24 hrs.	5 – 10	20 – 30
at 48 hrs.	50 – 100	100 – 200

There are great differences to be observed both in effectivities and effective doses according to the different pupal ages when the insects were treated. Though the males have got slightly bigger amounts than the females (owing to their smaller live weight), they seem more sensitive to FME.

b) In another experiment, 5th instar larvae of the bug, *Pyrrhocoris apterus* L. were treated topically in different ages after the last larval moult on the dorsal abdominal surface with different doses of dihydro-dichloro farnesenic acid methyl ester (DDFAME) in a standard 1 μl acetone solution. *Pyrrhocoris* has been found to be very sensitive to this special derivative of farnesenic acid (ROMANUK et al. 1967). The animals were kept at 28°C. After next moult we estimated the degrees of developmental injury as well as their ratios (percentages) to the total number of individuals.

The following main categories could be clearly distinguished:

D – insect died at moult,
0 – normal adult,
I – slightly injured adult with shortened elythrae;
V – VIth instar larva (superlarva).

The latter 3 categories are equal to those published by WILLIAMS and SLÁMA (1966). In this experiment we could not observe any other intermediate forms. The mortality of Vth instar larvae occurred before the last moult was not taken into consideration. We did not make any distinctions according to sex.

By calculating the *JH effectivity* from the viewpoint of reproductive activity (ability to mate and to lay fertile eggs), there was no need for an additional experi-

Table 6

The effect and effectivity of DDFAME treatment on Pyrrhocoris apterus in connection with the reproductive activity of adults

Age	Dosis of DDFAME in μg per spec.	No. of individuals		Number				Percentage				Effectivity of DDFAME treatment in %
		dead	living	of individuals in categories of JH effect								
		as L_5 larva		0	I	V	D	O	I	V	D	
6 hrs	0.0005	5	9	2	2	—	5	22	22	—	55	77
	0.005	0	13	3	4	—	6	23	31	—	46	77
	0.01	4	10	1	1	—	8	10	10	—	80	90
	0.05	3	11	—	—	—	11	—	—	—	100	100
	0.1	0	19	—	—	1	18	—	—	5	95	100
24 hrs	0.0005	3	6	6	—	—	—	100	—	—	—	0
	0.005	0	11	5	1	—	5	45	10	—	45	55
	0.01	0	12	1	3	—	8	8	26	—	66	92
48. hrs	0.001	2	11	11	—	—	—	100	—	—	—	0
	0.005	2	11	9	1	—	1	82	9	—	9	18
	0.01	0	14	5	2	—	7	36	14	—	50	64
	0.05	3	11	—	2	—	9	—	18	—	82	100
	0.1	1	14	—	—	1	13	—	—	7	93	100

ment in this case. The insects belonging to the categories I and V were not able to reproduce (ŽĎÁREK and SLÁMA, 1968). So the sums of percentages of individuals in categories I, V, and D gave us the effectivity of JH treatment (in percentage) in a definite experimental group, in connection with reproduction activity. The results are summerized in Table 6.

Taking 50% effectivity as a basis, the *effective dosis* ($ED_{JH}50$) of dihydro-dichloro farnesenic acid methyl ester for the larvae of *Pyrrhocoris apterus*, in connection with the reproductive activity of adults, falls below 0.0005 μg/spec. at 6 hrs., between 0.0005 and 0.005 μg/spec. at 24 hrs., resp. 0.005 and 0.01 μg/spec. at 48 hrs.

On this species we could observe the same change of JH sensitivity than on *Leptinotarsa*: the later the insects were treated, the higher doses could evoke the same effect, could have the same *effectivity*. It means: the *effective doses* which we have to apply to achieve a definite effectivity (e.g. 0.50 or 50%) rise with the age, in the case of these insect species.

Conclusions

In all these cases, the values of effectivity or effective dosis can satisfactorily characterize the effect of a juvenile hormone analogue on a special physiological activity of an insect pest, or on the rate of its survival. Therefore, one can take them into consideration from the viewpoint of practical use. Although these characteristics are valid only under the well defined conditions of our experiment, under standard circumstances, they could gain a more general significance.

What is the use of such investigations and calculations? In possession of these experimental data we are able to answer some questions of theoretical or practical importance. In this respect, the followings can be mentioned:

1. Drawing up dosis-effectivity curves;
2. comparing the effectivities of different treatments;
3. studying the influences of different ecological factors (e.g. temperature) on the effectivity of hormonal treatment;
4. establishing the duration of the sensitive period from the viewpoint of an economical JH treatment;
5. estimating, in a few exceptional cases, the doses to be used in the practice.

We hope to develop some still more accurate evaluation methods which can better describe the complex effects of juvenile hormone treatments sometimes encountered.

*

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Trophic Relations and Sex Meetings in Insects

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The periods of sexual and reproductive activity of insects have been shown to be determined by ecological factors which ensure the coincidence of hatching and trophic possibilities for the progeny. Two chains of mechanisms were assumed to ensure correlation between the trophic level and sexual activity: 1.) the presence of a right trophic level is a condition for the production of visual, auditory and chemical stimuli; 2.) the trophic level of the offspring ensures the concentration of both sexes, ensuring the preliminary condition of any meeting. These two assumptions have been tested by reviewing many data. The results obtained show that sex meeting cannot be regarded as an isolated phenomenon but as a link in a chain of events resulting in the production of the offspring in conditions consistent with its survival.

Unity in time and space is exceptional in insects reaching sexual maturity. Mating therefore implies sex meeting.

The problems connected with sex meeting have been studied very often. In insects tactile, visual, auditory and chemical senses are employed in the recognition of the sexes.

In his study of "*The Physiology of Insect Reproduction*", ENGELMANN (1970) reviews the various processes involved. Visual sex recognition generally operates only over distances of few meters due to the relatively poor visual acuity of insects. This applies to both diurnal and nocturnal species . . . "Emission and perception of sound appear to be superior to vision for distant recognition since vegetation is no obstacle. This mean of recognition becomes particularly effective in species which live low on the ground in grasslands. Chemicals act over greater distances than do either visual or auditory stimuli. In more recent years, insect species which emit attractants perceived by the opposite sex became rewarding research objects. As the number of reports incresases, it appears that sex attractants (pheromones) are to be found in very many species. This is the most common means of sex recognition over long and short distances".

These extracts from ENGELMANN's study draw special attention to the importance of pheromone production in sex meeting. In the entomological reviews of the past few years, very many studies have been centered on this aspect of insect ethology and on one of its applications: the use of sexual traps. The isolation

and synthesis of these pheromones have been undertaken, often successfully. The influence of age and physiological condition on insect pheromone production has been often studied.

On the other hand, ENGELMANN observes: "for many species the mating is one of the decisive factors which influence the total number of eggs a species lays. Mating, however, not only influences egg maturation as such, but also often stimulates oviposition".

Thus copulation not only provides the spermatozoa that fertilize the eggs and ensure the offspring of amphymixy species, but influences egglaying.

While copulation influences the sole emission of eggs, the inhibition of egglaying, prior to copulation, can only be effective when the female has the oviducts making egg-storage possible. The reason why copulation influences oviposition in certain species is that it very often stimulates not only emission but oogenesis (LABEYRIE, 1970a).

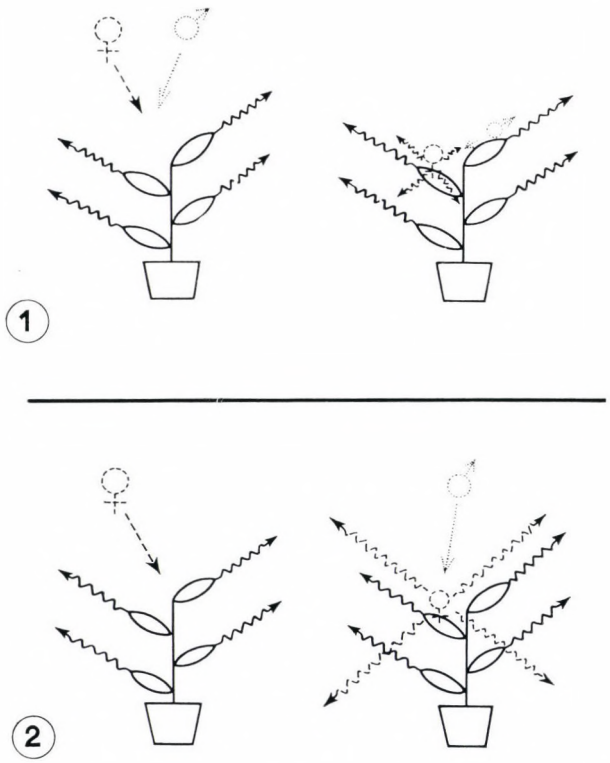

Fig. 1. The host plant attracts the two sexes, the attractiveness of the female is limited in space (1)

Fig. 2. The host plant attracts the female and induced production of the female pheromone (2)

As the trophic level does not, as a rule, represent a continuous area of useful supplies, the larvae must preferably be hatched in the presence of available food. So the oviposition behaviour often is adjusted to specific requirements and living conditions of the larvae (LABEYRIE, 1971). But, writes GHILAROV (1969) "only the more highly organized and specialized actively dispersing alate insects ensure the development of their progeny in appropriate conditions".

It is therefore not surprising that in many oliphagous insect species there is some correlation between the ovarial activity of females unable to store their occytes, and the egg-laying site.

But, insofar as copulation itself stimulates ovarial activity and egglaying, this stimulation can run contrary to the production of eggs in conditions favourable to their emission. It is a possibility that certain processes provide some connection between sexual activity and the trophic conditions favourable for the offspring.

This hypothesis can be proposed, since the periods of sexual and reproductive activity have already been shown to be determined by ecological factors achieving the coincidence in time of the hatching with the trophic possibilities of the environment.

Two chains of mechanisms ensuring correlation between the trophic level and sexual activity can be considered (Figure 1). The first hypothesis is that the presence of the right trophic level is a condition of the production of visual, auditory or chemical stimuli. The second hypothesis is that the trophic level of the offspring ensures the concentration of the sexes, the preliminary condition of any meeting (Figure 2).

These two hypotheses have been tested in various experiments.

Action of the Trophic Level on the Emission of Stimuli Making Sex Meeting Possible

RIDDIFORD and WILLIAMS (1967) have observed that in *Antheraea polyphemus* the females alone did not attract the males. "By contrast, 12 (33%) of the females mated in cages containing oak leaves; 10 (29%) mated when oak leaves were only in the vicinity of the cages . . . no mating occured among the 24 females subjected to the treatment with maple, birch, chestnut, elm, beech leaves", acceptable but not natural food plants of polyphemus silkworm. "Preliminary treatment of females (with oak leaves) for 4 to 6 hours was effective only when the treated females were promptly placed with males. A delay of only 30 minutes negated the effects of 24 hours of treatment".

RIDDIFORD (1967) has shown that trans-2-hexenal, a volatile compound found in oak leaves, stimulates the female to release its sex attractant. This com-

pound is perceived through the antennae, which transmit the stimuli inducing the production of the pheromone.

RAHN (1968) has studied the recapture of *Acrolepia assectella* males by sexual traps in an area where they had to compete with no native female. In several experiments the traps were placed respectively above bare ground, grassland, beets, and finally leek *(Allium porrum)*, the host plant of the caterpillars.

When the experiments were made in the beds of *Allium porrum*, 61% of the released males were recaptured, while in other conditions the percentage of recapture never exceeded 13%. In the experiments above fields in which leek and beets alternated, only the females placed above leeks attracted the males, even when they were farthest from the point where the males had been released.

The females of *A. assectella* remain attractive for 24 hours after the host-plant has been maintained for a day.

In the case of these two Lepidoptera, the adults do not feed on the plant, it is therefore on account of its nutritional value for the later forms of the insect, in other words also for the progeny, that the correlation became established.

This fact, the object of too few studies, is probably not exceptional. Thus SNOW and CALLAHAN (1967) observe that in *Heliothis zea* the presence of the host-plant increases the frequency of simple fecundations by 30% and that of double fecundations by 20%. MOREAU (1967) observes earlier matings in *Oscinella frit* in the presence of young plants of *Triticum*.

WOOD and BUSHING (1963) note that in *Ips confusus* "an association may exist between the production of the attractant and some nutritional requirement of the insect which is not satisfied by feeding on a non host species".

PITMAN (1969) observes that brevicomin obtained from the excreta of *Dendroctonus brevicomis* "has consistently given indifferent results or been totally ineffective. However, when a representative assortment of natural terpenes was tested in conjunction with brevicomin, 3-carene proved to be effective in activating the substance".

These few observations show the connection between the production of pheromones and the presence of the host plant. Couldn't the part played by the trophic level in the stimulation of sexual behaviour be the origin of the cases of nuptial gifts, involving the exchange of preys or food prior to copulation? For instance, in Heteroptera, *Stilbocoris natalensis* (CARAYON, 1963) the male offers the female a *Ficus* seed, and in *Panorpida harpobittacus* (BORNEMISSZA, 1964) the male alone captures the prey that the female eats during the copulation. Couldn't the establishment of the complex courtship of *Empididae* (FOULTON, 1913) be originally connected with the influence of the trophic level?

It would be equally interesting to find out whether the emission of auditory stimuli could not be induced by the presence of an environment favourable to reproduction. BUCK and BUCK (1966) have observed that the males of *Lampiridae*, *Pteroptyx malaccae*, found on certain *Sonneratia* along rivers in which the larval growth takes place, flash synchronously, which might attract the females.

Action of the Trophic Level on Sex Concentration

The males of *Acanthoscelides obtectus* can't discover the females unless they are within a radius of two centimeters. Sex meeting depends on the concentration of adults on a site favourable to oviposition. Experiments (LABEYRIE, 1970b) have shown that virgin females alone were incapable of attracting the males, by contrast the attraction is intense and rapid when the virgin females are placed with *Phaseolus* seeds, the natural environment of larval growth. The seeds alone are as attractive to the male as the seeds associated with females.

In the same way the females are attracted by seeds whether associated with the males or not, the latter having no attractive power by themselves. The trophic level in which the eggs are laid does not induce in this species the production of sexual stimuli, but ensures the gathering of adults which have thus a possibility of meeting each other. Now, this larval trophic level is not consumed by the adults.

PROKOPY (1968) has made similar observations in the case of *Rhagoletis pomonella:* "during the period when they were on the apples prior to mating, none of the individuals involved appeared to have extended their proboseis in search of food. After mating, most of the mated pairs flew to leaves or bark. The conclusion from these observations is that apples serve as a rendez-vous site for the sexes in mating activity . . . apples are not important as a feeding area but rather serve as a rendez-vous site for the sexes in mating activity and as the oviposition site".

ZWÖLFER (1969) observes a similar behaviour in other Trypetidae: "males and females of *Urophora siruna-seva* are attracted by the host plant . . . The localization of courtship and mating on the specific host plant has been also observed in other *Urophora* species" . . . "the localization of courtship and mating on the specific host plant appears to be important, because it provides a certain compensation for the lack of the specificity in the courtship behaviour. The existence of a host specific rendezvous in *U. siruna-seva* and *Urophora* species facilitates not only the encounter of males and females belonging to the same species, but operates also as an isolation mechanism preventing interspecific mating."

To ODHIAMBO (1968) "the familiar following swarms of males of certain species of *Glossina* which follow an individual host but do not necessarily feed on it may be an important method of recruitment of the two sexes for mating".

The concentrating part played by the trophic level may account for the fact that many insect species, in which the sexes are unable to perceive each other from a distance, are yet able to mate within a short time after imaginal moulting.

Conclusion

It is difficult to draw general conclusions from experiments involving but a few species, but the results already obtained show that sex meeting cannot be examined as an isolated phenomenon. Many ethological studies about sexual behaviour, which were made without taking ecological connections into account, ought to be resumed.

Sexual behaviour, and its preliminary condition, sex meeting, are but links in a chain of events resulting in the emission of the offspring in conditions consistent with its survival. In so far as the condition a final event connected with the presence of a given trophic level, it would be surprising that selective processes should not have brought about the association of sex meeting with the presence of this environment. Many instances are very likely to show that the cases we have reviewed are not exceptional.

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Der Einfluß der Wirtspflanze und der Nichtwirtspflanzen auf Eibildung und Eiablage der Rübenmotte *Scrobipalpa ocellatella* Boyd *(Lepidoptera, Gelechiidae)*

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Oogenesis and egglaying are governed by stimuli, in case of the sugar-beet moth *Scrobipalpa ocellatella* Boyd, originating from sugar beet. The presence of sugar-beet (or its aqueous extract) stimulates the forming and laying of eggs and determines at the same time the site of egglaying as well. In absence of the host plant the egg formation decreases considerably. The stimuli seem to be of chemical character, the stimulant is water soluble and not very volatile. All extracts made from non-host plants had a repellent action on female moths and did not stimulate the oogenesis. The repellent action of non-host plants simultaneously with the marked attractant effect of sugar beet leads the females for oviposition soon to the beet cultures. The proper selection of egglaying sites provides the ecological factor beneficial for young larvae and contributes to the preservation of the species in a given area. In this way also the rapid colonization of *S. ocellatella* into sugar beet plantations may be explained.

Die Rübenmotte ist ein oligophager Kleinschmetterling, dessen Raupen sich nur auf Pflanzen der botanischen Gattung *Beta* (Chenopodiaceae) entwickeln, und zwar auf: *B. vulgaris*, *B. trygina* und *B. maritima*. Die Schmetterlinge haben keine Nahrungsbeziehungen mit der Rübe *(B. vulgaris)*. Sie ernähren sich von Wasser und Zuckerwasser.

Die Auswahl der Wirtspflanze ist vor allem durch die Weibchen während der Eiablageaktivität ausgeführt. Die Erforschungen der Beziehungen zwischen Wirtspflanze und Schmetterling werden also bei diesem Insekt nicht durch die Nahrungsaufnahme kompliziert. Wir erforschten zunächst die Beziehungen zwischen den Weibchen und der Rübe. Erst in jüngster Zeit haben wir mit der Analyse der Reaktionen der Weibchen gegenüber der Nichtwirtspflanzen begonnen.

A — Einfluß der Wirtspflanze auf die Eibildung und die Eiablage

Wir haben in früheren Veröffentlichungen (Robert, 1965, 1970) ausgezeigt, daß

1) die Rübe oder ein Wasserextrakt dieser Pflanze bei allen Weibchen das Eiablageverhalten auslöst. Die Fruchtbarkeit beträgt dann 180 bis 200 Eier pro

Weibchen. Fehlen Rübe oder Rübenextrakt, so legen 40 bis 70% der Weibchen überhaupt nicht, während die anderen im Durchschnitt nur 30–50 Eier legen (Abb. 1);

2) die Eier auf Rüben oder auf mit Rübenextrakt impregnierten Unterlagen abgelegt werden. Die neutralen Unterlagen bleiben unbeachtet (Tabelle 1);

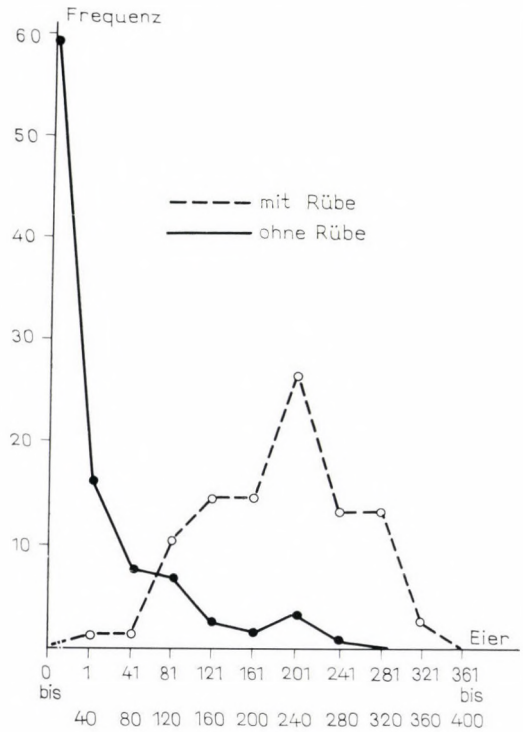

Abb. 1. Frequenz in Prozent der eilegenden Weibchen (die Zahlen der abgelegten Eier sind in Klassen von 40 eingeteilt) Alle mit Rüben versorgten Weibchen legen Eier ab. In Abwesenheit der Rübe sind die Reaktionen der Population heterogen; die Mehrheit der Weibchen, hier 60%, legen keine Eier, einige hingegen legen Eier

3) die Anwesenheit der Rübe oder des Rübenextrakts die Physiologie der Eiröhrenfunktion beeinflußt; sie stimuliert die Ovogenese so, daß die Reifung der Ovocyten beschleunigt wird. Im Gegensatz dazu verlangsamt sich die Ovogenese bei Abwesenheit der Wirtspflanze. Aber nach einer langen Abwesenheit der Rübe, stimuliert ein Auffinden der Wirtspflanze die Ovogenese und die Eiablage fängt an (Abb. 2).

Der Wirt übt also hier einen unmittelbaren stimulierenden Einfluß auf die Eiröhre aus, ohne daß ein Nahrungseinfluß vorhanden ist. LABEYRIE (1960a, b)

machte ähnliche Beobachtungen bei einem schmarotzenden Hymenoptera *Diadromus* spec. und bei einem phytophagen Coleoptera *Acanthoscelides obtectus*.

4) die Reizfaktoren der Rübe chemischer Natur sind. Die Wirkstoffe sind wasserlöslich und wirken hauptsächlich bei Kontakt.

B — Einfluß der Nichtwirtspflanzen

Die Schmetterlinge schlüpfen im Frühling aus Feldern, die im Vorjahr Rüben trugen. Während der Suche nach einem neuen Rübenacker kommen die Weibchen mit vielerlei Pflanzen in Berührung, die nicht dazu geeignet sind, das Überleben der Nachkommenschaft zu sichern.

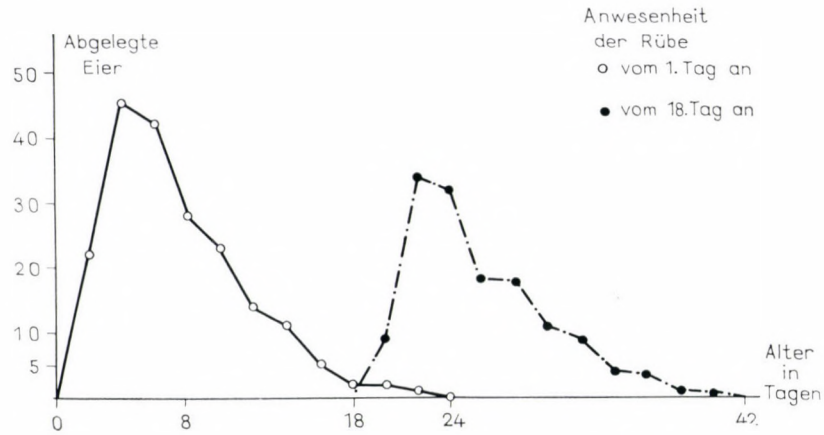

Abb. 2. Selbst bei verzögerter Auffindung der Wirtspflanze bleibt die Eiablage möglich. Beider mit Rüben vom ersten Tagen versorgten Weibchen setzt die Eiablage sofort ein, und erstreckt sich auf 20 bis 25 Tage; dann gehen die Weibchen ein. Wird die Rübe 18 Tage nach dem Schlüpfen geboten, so ist die Eiablage zuerst schwach; sie steigt dann nach der Stimulierung der Ovogenese durch die Wirtspflanze und währt noch 20 Tage. Die Lebensdauer der Weibchen ist verlängert

Der Einfluß der Nichtwirtspflanzen wurde vor allem bei Insekten im Stadium der Nahrungsaufnahme studiert. (JERMY 1958, 1966, GUPTA und THORNSTEINSON 1960a, HSIAO und FRAENKEL 1968a). Nur wenige Studien befassen sich mit Reaktionen der eierlegenden Weibchen den Nichtwirtspflanzen gegenüber. Die Untersuchungen von GUPTA und THORNSTEINSON (1960b) bei *Plutella maculipennis* und die von HSIAO und FRAENKEL (1968b) bei *Leptinotarsa decemlineata* haben gezeigt, daß Nichtwirtspflanzen eine abstoßende Wirkung auf die eilegenden Weibchen haben.

Material und Methode

Der attraktive oder abstoßende Einfluß der Nichtwirtspflanze ist bei den eierlegenden Weibchen mit Hilfe von Pflanzenextrakten studiert. Bisher wurden 7 Pflanzen von verschiedenen botanischen Familien geprüft (Tabelle 1).

Von diesen Pflanzen werden Extrakte mit Wasser oder mit Cyclohexan hergestellt. Getrocknete Blätter werden pulverisiert und in Wasser oder Cyclohexan eingeweicht.

Der Cyclohexanextrakt wird lediglich filtriert.

Bei der Gewinnung des Wasserextraktes gehen wir wie bei der Gewinnung des Rübenextraktes (ROBERT, 1968) vor; vier Teile Azeton werden mit einem Teil Wasserextrakt gemischt, der Niederschlag wird entfernt, das Azeton anschließend verdunstet.

Die Konzentration dieser Lösung ist nahezu der Dichte des Pflanzensaftes gleich.

Mit Hilfe von Gelatine klebt man Schmirgelpulver auf neutrale Papierstreifen (70 × 10 mm) um den Insekten die nötige Rauheit zur Eiablage zu bieten. Diese Papierstreifen werden in die Extraktlösungen getaucht. Dann läßt man das Lösungsmittel bis zur gänzlichen Austrocknung verdunsten. Zeugenstreifen werden mit Schmirgelpulver beklebt, in reines Lösungsmittel getaucht und getrocknet.

Drei mit nur einem Pflanzenextrakt behandelte Streifen und drei Zeugenstreifen werden in einem zylindrischen Versuchskäfig von 20 cm Höhe und 20 cm Durchmesser aufgehängt. In jeden Käfig werden 6 Weibchen und 8 Männchen eingesetzt. Die Eiablage wird 7 Tage lang beobachtet.

Jedes Experiment wurde viermal wiederholt

Ergebnisse

Die abgelegten Eier stammen von jener Minderheit der Weibchen, die auch bei Abwesenheit der Wirtspflanze legen, das heißt 30 bis 40 Prozent.

Die Ergebnisse sind in Tabelle 1 angegeben.

1) Der Wasserextrakt der Rübe wirkt attraktiv, der Cyclohexanextrakt bleibt ohne Wirkung.

2) Alle Extrakte aus Nichtwirtspflanzen wirken abstoßend (repellent). Die Cyclohexanextrakte aus Kartoffelkraut und besonders die aus Weinblättern haben die stärkste Wirkung.

3) In einem anderen Versuch haben wir schon mit Rübenextrakt behandelte Papierstreifen mit Robinienextrakt nachbehandelt. In diesem Falle verdeckt der Robinienextrakt merklich die anziehende Wirkung der Wirtspflanze (Tabelle 2).

4) Die Extrakte von Nichtwirtspflanzen stimulieren die Eibildung nicht.

Tabelle 1

Einfluß verschiedener Pflanzenextrakte auf die Lokalisierung der Eiablage

	Wasserextrakte			Cyclohexanextrakte		
	Summe der abgelegten Eier	Abgelegte Eier (in Prozent)		Summe der abgelegten Eier	Abgelegte Eier (in Prozent)	
		behandelte Unterlage	Zeugen-unterlage		behandelte Unterlage	Zeugen-unterlage
Wirtspflanze *Beta vulgaris* L.	4081	86	14	1696	49	51
Nichtwirtspflanze						
Solanum tuberosum	747	25	75	940	4	96
Daucus sativus Hoffm.	1456	24	76			
Medicago sativa L.	366	22	78	765	29	71
Carpinus betulus L.	669	15	85			
Robinia pseudoacacia L.	1035	15	85	1557	28	72
Zea Mays L.	977	12	78	1264	17	83
Vitis vinifera L.	542	9	91	1394	0.07	99.93

Tabelle 2

Einfluß der Pflanzenextrakte auf die Lokalisierung der Eiablage

Wasserextrakte	Summe der abgelegten-Eier	Abgelegte Eier (in Prozent)	
		behandelte Unterlage	Zeugen-unterlage
B. vulgaris	4081	86	14
B. vulgaris + *R. pseudo-acacia*	1214	43	57
R. pseudoacacia	1035	15	85

Diskussion

1) Der Wasserextrakt der Rüben hat eine sehr spezifische attraktive Wirkung auf die eierlegenden Weibchen. Der Cyclohexanextrakt der Rübe enthält weder attraktive noch abstoßende Faktoren.

Alle Extrakte aus Nichtwirtspflanzen wirken abstoßend. Die aktiven Stoffe befinden sich also sowohl in den Wasserextrakten als auch in den Cyclohexanextrakten. Demnach reagieren die Weibchen auf verschiedene Substanzen von unterschiedlicher chemischer Beschaffenheit negativ.

Die enge Spezialisierung bei der Wahl der Unterlage für die Eiablage ist die Folge von zwei Verhaltensarten: einerseits positive Reaktionen auf spezifische Wirkstoffe, anderseits negative Reaktionen auf die vielfältigen unterschiedlichen Wirkstoffe der Nichtwirtspflanzen. Ähnliche Ergebnisse haben die Untersuchungen

über die Nahrungswahl kauender Insekten erbracht (JERMY, 1966, HSIAO und FRAENKEL, 1968a).

2) Die Fähigkeit der Chemorezeptoren, zahlreiche, unverwandte Reize zu empfinden und wahrzunehmen ist äußerst bemerkenswert und wirft Fragen über die Physiologie dieser Sinnesorgane auf.

3) Es zeigte sich, daß die abstoßenden Stoffe die Anziehungskraft des Rübenextraktes zumindest teilweise überdecken können. Könnte diese Möglichkeit der Anfang für eine spezifische Pflanzenschutzmethode sein?

4) Die Beziehungen zwischen den Weibchen der Rübenmotte und der Pflanzenwelt bewirken Verhaltensweisen und physiologische Reaktionen, die beträchtliche ökologische Folgen haben:

a) Die abstoßenden Reaktionen auf die Nichtwirtspflanzen und die anziehende Wirkung der Wirtspflanze führen die eierlegenden Weibchen fast ausschließlich auf die Rübe. Diese genaue Wahl der Unterlage für die Eiablage ist ein ökologischer Faktor, der für die Ernährung der jungen Raupen äußerst günstig und somit für die Erhaltung und Entwicklung der Art wichtig ist.

b) Bei Abwesenheit der Wirtspflanze ist die Eiablage gedroselt, die Eibildung verlangsamt und die Lebensdauer verlängert. Das Insekt kann unter diesen Umständen, fast ohne Eier abzulegen, seine Wirtspflanze während einer langen Periode, 20 bis 30 Tage, suchen und so wenigstens 50 bis 70 % seines Fortpflanzungspotentials erhalten. Der Eibildungs- und Eiablageprozeß wird erst beim Auffinden der Rübe ausgelöst. Diese Fähigkeiten sind sehr günstig für die Art, wenn die Wirtspflanze selten oder weit entfernt ist, jedoch ungünstig für den Bauern.

c) Im Gegensatz dazu ist bei fortlaufender Anwesenheit der Rübe die Eireifung intensiv und die Eiablage ausgelöst. Diese Verhältnisse sind ständig in den Rübenfeldern verwirklicht und bewirken das höchste Fortpflanzungspotential. So kann man die außerordentliche Geschwindigkeit der Ansiedlung der Rübenfelder durch die Rübenmotte erklären.

Die Reaktionen der Weibchen auf verschiedene chemische Pflanzensignale erlauben es, daß die Fortpflanzungsaktivität den Ausbeutungsmöglichkeiten der Umwelt für die Nachkommenschaft angepaßt werden kann.

Diese bei der Rübenmotte gewonnenen Ergebnisse können wahrscheinlich nicht verallgemeinert werden. Aber es scheint mehr und mehr festzustehen, daß sensorielle Beziehungen zwischen Insekten und Pflanzen unter verschiedenen Aspekten in der Natur verbreitet sind. Es bleibt zu wünschen, daß mehr Forschungen in dieser Richtung unternommen werden.

Zusammenfassung

Die Eiablage und Eibildung sind bei der Rübenmotte durch Reizfaktoren ausgelöst, die aus der Zuckerrübe stammen; die Anwesenheit von Beta-Rüben (oder deren Wasserextrakt) stimuliert die Eibildung, Eiablage und determiniert gleich-

zeitig die Lage der Eier auf der Pflanze. Die Oogenese geht in Abwesenheit der Wirtspflanze bemerkbar zurück. Die Reizfaktoren sind von chemischer Natur zu sein, sind wasserlöslich und wenig flüchtig. Alle Extrakte dagegen aus Nichtwirtspflanzen wirken abstoßend für die Weibchen und stimulieren auch die Eibildung nicht.

Die abstoßenden Reaktionen auf die Nichtwirtspflanzen und die anziehende Wirkung der Wirtspflanze führen die eierlegenden Weibchen fast ausschließlich auf die Rübe. Diese genaue Wahl der Unterlage für die Eiablage ist ein ökologischer Faktor, der für die jungen Raupen günstig und somit für die Erhaltung und Entwicklung der Art wichtig ist. So kann auch die außerordentliche Geschwindigkeit der Ansiedlung der Rübenfelder durch den Schädling erklärt werden.

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Study of Factors Influencing the Fecundity and Fertility of Codling Moth (*Laspeyresia pomonella* L., Lepid.; *Tortr.*)

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Most of the experimental work has been carried out on a codling moth population originating from Yakima (U.S.A.). It has been stated that there was a correlation between the body weight and the number of eggs laid per female ($r = 0.677$); the quality of larval food caused greater differences in the body-weight of the females (12.2 mg) than in that of the males (3.1 mg). However, the extent of egg production did not correlate to the number of viable eggs. The number of inviable eggs increased towards the end of the egglaying process of individual females. The number of these eggs did not correlate to the age of females, length of egglaying period, weight of male, etc. although, compared to the total number of eggs laid, the percentage of inviable eggs was greater in case of heavier females and in case of consecutive matings of one male. In this last case the percentage of inviable eggs could be artificially decreased by applying FME topically on the male before the fourth mating.

The codling moth (*Laspeyresia pomonella* L.) may be regarded as the most injourious pest of Hungarian apple orchards. It has two generations yearly and overwinters inside a cocoon as last instar larva. The pupation takes place the following spring. The females lay their eggs singly on the apples.

The sterile male program for the codling moth in Hungary (JERMY and NAGY, 1969, 1970), made it necessary to investigate the factors wich influence reproduction in this species. In our studies we aimed to get answers to two questions: 1) What factors influence the fecundity of females? 2) What factors influence the number of viable eggs laid per female?

Methods and Materials

The experiments were carried out with a laboratory population originating from Yakima (Washington, U.S.A., courtesy of Dr. B. BUTT).

The larvae were reared in green apples previously stored at low temperature. The rearings were at 23–28°C. The larvae spun their cocoons inside corrugated paper strips placed into the massrearings; the paper strips were transferred into hygrostates where the pupation took place. The newly emerged adults were removed twice daily and the females were kept singly at 23°C for 24 hours. Virgin

females could be distinguished from mated ones, as the latter soon began to lay eggs on the glass walls of the vials.

The adults in the experiments were kept in jars containing wet cotton wads. The cotton wads were replaced every second day. The females were dissected after death for determining the spermatophores inside their bursae copulatrices.

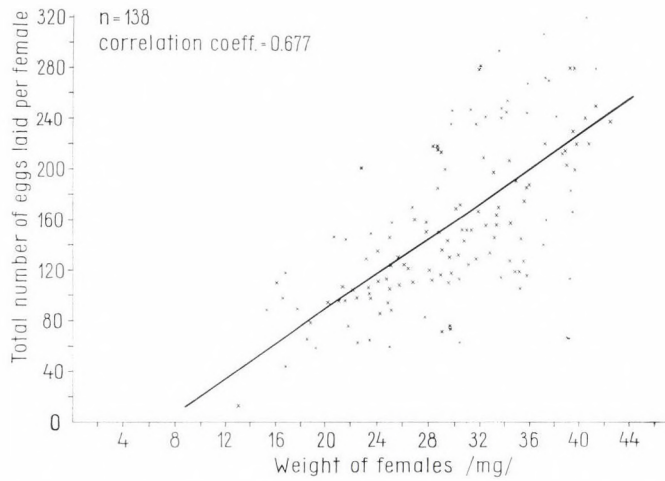

Fig. 1. Relationship of female weight and total number of eggs laid by a female of codling moth

Results and Discussion

1. *Factors influencing the fecundity of codling moth*

Different constant temperatures acting throughout the larval and pupal development (18, 23, 28°C) did not seem to affect the number of follicles or the number of eggs actually laid (DESEŐ, 1970). Contrary to this, the importance of temperature prevailing during the egg laying period was shown (KLINGLER, VOGEL and WILLE, 1958). It was observed in the field that with increasing temperature at dusk, the number of eggs laid on the fruits increases proportionally. The positive affect of some other factors, such as nutrition (honey-water and water) was reported by WIESMANN (1935), and mating was reported by SCHMID, (1968, in BENZ, 1970) in the case of the laboratory strain "Maag" and "Waedenswill" in Switzerland.

It is wellknown for many insects, including some lepidopterous species that amount and quality of larval food determines the egg production of the adults (SOLOMON, 1967, MANI, 1968). The linear correlation between pupal weight and number of eggs laid of the adults were reported for example for *Lymantria monacha* L. (ZWÖLFER, 1933).

Table 1

Weight data of codling moth adults reared in green apples of different quality

Type of larval nutrition	Mean weight of 100 adults (mg)		
	Females	Males	Average
Reared in stored green apples in late spring	24.2	17.6	20.9
Reared in fresh green apples in late spring	36.4	20.7	28.5

We also studied the correlation between the weight of females and number of eggs laid in the codling moth, fed water only (Fig 1).

As shown, there was a linear correlation between the two parameters, but the data show a relatively great variability. The average weight of females was 29.4 mg, the average number of eggs laid was 159.8 per female.

However, it turned out at the end of winter that the number of eggs laid became lower than in spring. We attributed this to changes in food value of the green apples during the winter storage.

In Table 1 data may be found regarding the effect of shrunken apples at late spring and that of fresh green apples in late spring on the weight of females and males. There are great differences in the average weights especially in the females (40%). It is to be mentioned that the difference in the weight of males is not so great (24%).

2. *Factors influencing the fertility of codling moth*

In Fig. 1 the number of eggs laid was related to the weight of the females. Although the fecundity of females increases with increasing weight, nearly always a percentage of the eggs laid is inviable. No correlation was found between the percentage of inviable eggs and the weight of the females or those of the males in the same rearing jar. There was also no correlation between the percentage of inviable eggs laid and the speed of the egglaying process.

If however the rhythm of appearance of inviable eggs was studied in relation to the whole oviposition process, we observed that at the beginning the per-

centage of inviable eggs laid was quite low. Some examples of increasing inviability during the egglaying are given in Fig. 2.

An increase in inviable eggs occurs independently of the total number of eggs laid always towards the end of the egglaying process. Among these inviable

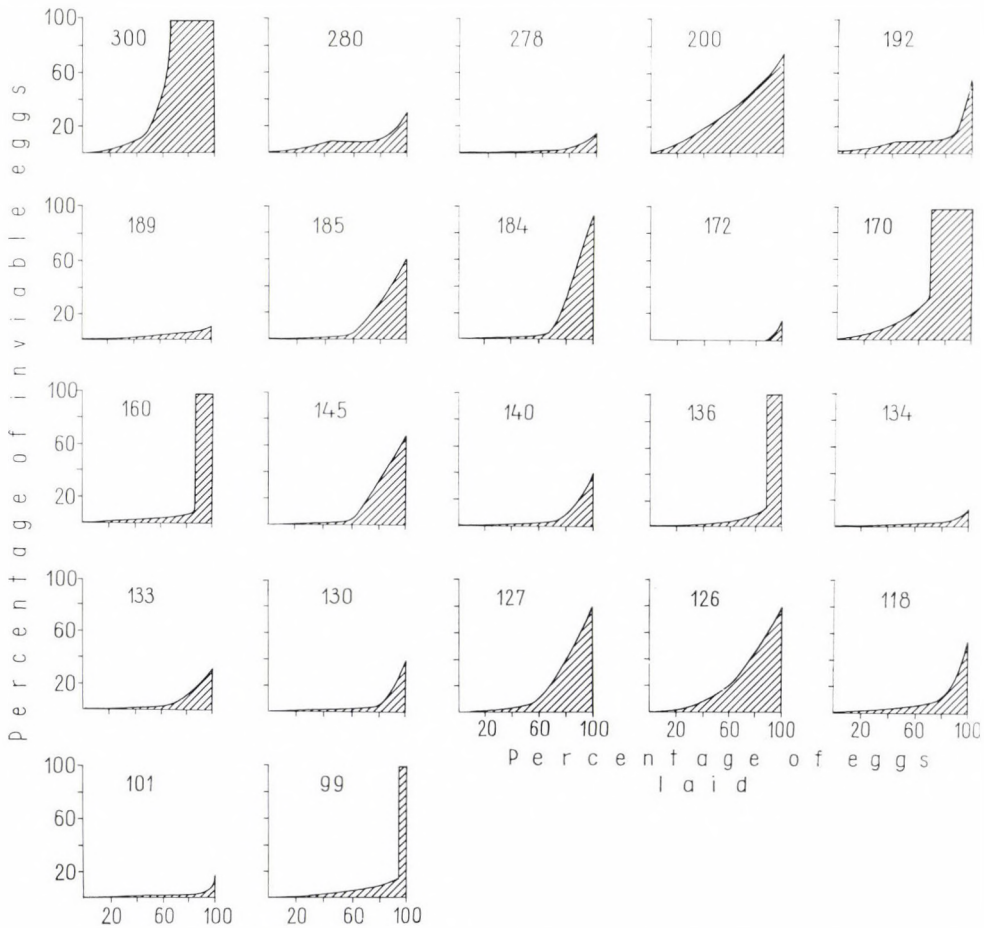

Fig. 2. Increase in the percentage of inviable eggs in course of the egg-laying period. (Inviable eggs: dark area.) The number in brackets indicates in each diagram the total number of eggs laid. Ordinate: Percentage of inviable eggs. Abscissa: Percentage of eggs laid

eggs many eggs were found in which the embryogenesis had proceeded to different degrees and also many eggs which did not develop at all. This was observed not only in the laboratory population of Yakima, but also in native Hungarian populations, or even in cases when the female mated repeatedly.

The exact causes for the impaired embryonic development are not known, but it could be conjectured that the eggs laid later during the oviposition period had obtained inadequeate maternal nutrition as was suggested for *Malacosoma pluviale* (WELLINGTON, 1965), and for other insects as well (see LABEYRIE, 1968). On the other hand, the observed increase of sterile eggs in the bean weevil, *Acanthoscelides obtectus* SAY., towards the end of egglaying was attributed to the malfunctioning of the male (HUIGNARD, 1969).

Table 2

Percentage of inviable eggs laid by females lighter and heavier than the average weight

Percentage of inviable eggs	Number and percentage of females			
	lighter than 29 mg		heavier than 29 mg	
0— 2	11	37%	5	10%
3—20	9	30%	9	18%
21—40	9	30%	26	52%
41—60	0	—	8	16%
60<	1	3%	2	4%

In Table 2 the percentage of inviable eggs is shown, laid by females lighter and heavier than average. It can be seen that the percentage of inviable eggs stayed under 20% in 67% of the cases in the group of females lighter than the average. In the cases of the heavier females the percentages of inviable eggs were several times higher. It may be concluded therefore that a low amount of reserves in the females does not increase the number of inviable eggs laid. We may then assume that possibly the males play a role in influencing the percentage of inviable eggs laid. This was then further investigated, as follows.

In two experiments, which differed from each other only in a little measure, the number and size of spermatophores produced by one male, was investigated. In Table 3 we summarized the number and size of spermatophores, which were found in the bursae copulatrices of the females, kept in aggregation of 1 male and 10 females. In Table 4 are shown the changes in the size of spermatophores from consecutive copulations. In this case the male got a virgin female only then, when the earlier female had begun to oviposit.

We can conclude (Tables 3 and 4) that in the case when the male could choose the partner, the spermatophores were more complete than in the other case. In the latter cases it was often found that the walls of the spermatophore were soft and it was impossible to measure them. The reasons for this are not known, but the possibility exist of an influence of the permanent presence of many virgin females, i.e. the effect of the female sex-pheromone.

As shown in Tables 3 and 4 a male may mate up to eight times. During one copulation more than one spermatophore may be formed. In Table 3 the male

Table 3

Number and size of spermatophores in the females of codling

No. of male	Weight (mg)	Number and size of spermatophores			
		♀1	♀2	♀3	♀4
1	20.2	2.0×1.5	1.8×1.5	1.8×1.2	1.8×1.2 1.5×1.2
2	21.3	2.0×1.2	1.8×1.5	0.8×0.8	0.5×0.5
3	20.6	2.5×1.2 2.5×1.0 2.0×1.2 0.5×0.5	1.8×1.2	0.8×0.5 0.5×0.5 0.5×0.4 0.3×0.2 0.3×0.2	0.5×0.5 mass
4	19.6	2.0×1.2	1.8×1.2 mass	1.8×1.0 1.4×1.0	1.2×1.0
5		2.0×1.5	0.5×0.5	0.5×0.5	—
6		2.2×1.5	2.0×1.0	1.5×1.2	1.0×1.2
7	16.2	2.0×1.2	0.8×0.5	0.8×0.5	0.4×0.5
8	14.0	2.5×1.2	0.5×0.5	mass	—
9	15.7	2.0×1.0	1.8×1.5	1.8×1.5	1.8×1.2
10	17.5	2.2×2.0	2.1×1.2	mass	—
11	17.4	2.0×1.2	2.0×1.0	1.2×1.0	mass
12	21.4	2.0×1.5	2.0×1.0	1.5×0.8	1.0×0.8

* The first number indicates the widest, the second the longest measure of the spermatophore, without the neck.

** All eggs laid were sterile.

No. 12 is an example for natural occurrence of a sterile male in the Yakima population.

The changes in the size of spermatophores from consecutive copulations are shown in Table 4, i.e. the size of spermatophores decreased. The walls of spermatophores became thinner, usually from the third copulation on. At the last matings only a shapeless mass could be found in the bursae copulatrices. In these latter cases one could not determine the role of the secretion of the bursa copulatrix in digestion of the outer wall of the spermatophore.

The effect of consecutive matings of individual males on the fecundity and fertility of the females mated once only, was studied as follows.

As shown in Table 5 there is no significant difference in the fecundity of females following the consecutive matings of the male. That means, the total number of eggs laid by a female is independent of the number of copulations of the male (however, after FME the amount of eggs laid is somewhat lower).

There is no significant difference in the percentage of inviable eggs laid after the first and second matings, but after the third and fourth copulation this percentage increases significantly (Table 5). One may assume that with the decreas-

moth (1 male and 10 females in every cage)

in one female (mm)*					Number of eggs laid
♀5	♀6	♀7	♀8	♀9–10	
1.5×1.2	0.8×0.5 0.5×0.3	0.8×0.5 mass	mass	—	857
0.5×0.3	mass	—	—	—	691
mass	—	—	—	—	668
0.5×0.5 0.5×0.3	mass	—	—	—	703
—	—	—	—	—	549
0.5×0.3	—	—	—	—	615
—	—	—	—	—	550
—	—	—	—	—	497
1.8×1.0	1.5×1.0	0.7×0.4	0.5×0.3	—	250
—	—	—	—	—	350
—	—	—	—	—	330
mass	—	—	—	—	286**

ing size of spermatophores the ratio of inviable eggs to total number of eggs laid, increases. This may be the consequence of the change in the ratio of eupyren and apyren spermatozoa but it may also be the consequence of a decreased viability of sperm following the inadequate functioning of the accessory sex glands in these males.

Table 5

Consecutive matings of males with virgin females and their effect on egglaying

		Number of females	Number of eggs laid	Inviable eggs (percent of total)	Significance t-test
1st mating		37	112±16*	17.6±2.0	$p < 1\%$
2nd mating		36	114±11	20 3±2.3	
3rd mating		20	131±14	26.1±3.4	
4th mating	Check	23	118±15	34.6±3.9	$p < 1\%$
	FME (1 μg/1♂)	9	86±17	18.5±3.3	

* Mean ± S. E.

Table 4

Number and size of spermatophores of

No. of male	Weight (mg)	Number and size of spermatophores			
		♀1	♀2	♀3	♀4
1	20.7	—	2.0×1.8	—	—
2	11.3	—	—	2.0×1.8 1.8×1.5	—
3	18.5	2.0×1.7 1.0×1.0	1.0×1.2	soft	0.5×0.8 0.5×0.3
4	13.8	—	—	—	—
5	15.0	—	2.0×1.0	soft	1.0×0.8 0.8×0.5
6	19.3	—	2.0×1.2	1.0×0.5 0.8×0.5 0.5×0.8	soft
7	15.9	2.0×1.2	0.8×0.5 0.8×0.5 0.8×0.5 0.8×0.5	soft	mass
8	17.0	—	2.0×1.3	—	—
9	17.1	—	2.0×1.0	—	1.8×1.5
10	26.3	—	2.0×1.5	soft	—
11	18.0	1.8×0.8	1.5×1.0	soft	soft
12	14.2	2.0×1.2	0.5×0.5 0.5×0.5 0.5×0.5 0.5×0.5	0.8×0.5	—
13	20.2	—	1.8×1.0 0.8×0.8 0.8×0.5	—	2.0×1.2
14	18.2	2.0×1.5	1.8×0.8	0.8×0.5 0.5×0.3	mass

* The first number indicates the widest, the second the longest measure of the spermatophore, without the neck.

It is well known that in some cases juvenile hormone influences the functioning of the male accessory glands (see in ENGELMANN, 1970). In a preliminary study 1 μg farnesylmethyl-ether (synthetized in the Dept. of Chem. of the Inst. for Plant Protection, Budapest) dissolved in acetone was applied topically to males after the third copulation. As is shown in Table 5, the percentage of inviable eggs laid by females mated to these males decreased and reached approxi-

individual males in consecutive matings

consecutive matings					
♀5	♀6	♀7	♀8	♀9	♀10
soft	1.2×1.0	1.2×0.4 0.5×0.8	soft	0.5×0.1	—
mass	mass	—	—	—	—
mass	mass	—	mass	—	—
mass	mass	—	—	—	—
mass	mass	mass	—	—	—
1.0×1.8	soft	—	—	—	—
—	1.2×1.2	mass	—	—	—
—	1.0×1.3 0.5×0.3	0.8×0.8	—	—	—
—	0.7×1.4	—	—	—	—
—	mass	mass	—	—	—
soft	mass	mass	—	—	—
—	—	—	—	—	—
mass	—	1.0×0.8	—	—	—
1.0×1.0 soft	mass	—	—	mass	—

mately the same level as after the first mating. The question, whether that is an effect on the general metabolism, or whether FME influences only some of the accessory glands, or whether, the ratio of the eupyren and apyren spermatozoa is affected by the juvenile hormone analogue, remains open.

Conclusions

1. The fecundity of the codling moth female depends primarily on her weight. The quality of the larval nutrition influences the weight of female stronger than that of the male.

2. One male may mate up to eight times. During one copulation more than one spermatophore may be formed. The size of spermatophores decreases in consecutive matings. The walls become very thin. After the later copulations only a shapeless mass is found in the bursae copulatrices.

3. The percentage of inviable eggs in the first and second mating of the same male is about 20%.

4. After the third and fourth mating the percentage of inviable eggs increases significantly.

5. The topical application of farnesylmethyl-ether after the third mating again decreases the percentage of inviable eggs to the level of the first mating.

6. The consecutive matings have no influence on the total number of eggs laid per female.

*

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Biological Background and Outlook of the Antifeedant Approach to Insect Control

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The use of antifeedants for population suppression in phytophagous insects is based on the following: (1) feeding inhibitors play a decisive role in the host selection; (2) the central inhibitory state of the nervous system induced by inhibitory stimuli has a long decay time, and in most cases induces locomotion; (3) reduction of food intake and disturbing of normal feeding rhythm caused by the presence of an antifeedant results in slowing down of development, and in decreasing of reproduction rate; (4) adaptation to inhibitory stimuli is unlikely; (5) compounds of very different chemical structure can inhibit feeding which strongly increases the probability to find compounds adequate for practical purposes. However, the requirements to be met by a suitable antifeedant are quite numerous, so that much more research is needed in this field.

The behaviour of insects is related to very specific signals of their environment. It is, therefore, logical to suppose that the normal pattern of behaviour, and at the same time, the ontogenesis and the dynamics of a certain insect population can be influenced by manipulating some specific environmental signals to which the receptors of the insect are tuned.

On the other hand, the specificity of these signals renders it possible to find methods of changing certain signals vital for an insect pest without affecting any other members of the biocenose by this procedure. That means highly selective methods of control which is the aim of research for new ways replacing the use of conventional insecticides.

One of the groups of specific signals which could be used for such purposes is that of the chemical stimuli governing host selection and food intake in phytophagous insects.

The Behavioural Basis of the Use of Antifeedants

The role of inhibitory stimuli in host selection. Since VERSCHAFFELT's (1910) classical experiments carried out on caterpillars sixty years ago, it was supposed for a long time that host specificity is due to the adaptedness of the phytophagous insects to specific feeding stimuli (phagostimulants) represented by specific secondary plant substances e.g., glucosides, alkaloids, etc. Later DETHIER (1953) suppos-

ed that the host specificity of oligophagous insects was determined by their feeding reaction to secondary plant substances while the host range of polyphagous insects was due to the occurrence of substances inhibiting feeding on the non-host plants. However, investigations carried out in the late 50s have clearly shown that feeding inhibitors play a decisive role in the host selection of both oligo- and polyphagous species (JERMY, 1958, 1961a, GUPTA and THORSTEINSON, 1960). This statement has been proved by several other experiments carried out during the last decade (JERMY, 1966, MA, 1969).

The increasing number of oligo- and polyphagous insects which can be reared on synthetic diets not containing specific (secondary) plant substances directly proves that the latter, as specific feeding stimulants, have no vital importance in the feeding behaviour and food specificity of these insects.

The importance of feeding inhibitors in determining food selection means that feeding of the insects can be inhibited even on their most preferred host plants containing an optimal combination of feeding stimuli, by applying suitable feeding deterrents.

The inhibitory state of coordinating centres induced by inhibitory stimuli. DETHIER, SOLOMON and TURNER (1965) have shown that a water-satiated blow fly, *Phormia regina*, does not extend its proboscis, which is the first step to begin feeding, when water touches a labellar hair. However, if another labellar hair is stimulated by sucrose directly prior to water stimulation, water stimulation causes proboscis extension. This induced responsiveness to water reflects a persisting central nervous system excitatory state (CES) which decays after a time.

A similar CES can be found in chewing phytophagous insects. Starving Colorado potato beetle adults walking outdoors on an indifferent surface keep a more or less straight direction as a result of light-compass orientation. Putting a potato leaf close to the beetle so that it gets olfactory stimulation from it, then slowly moving the leaf away from the beetle, the latter would first stop walking and then would follow the leaf. When, at that moment, the leaf is suddenly taken away, the beetle would stop again, would make "searching" movements on the spot ("dancing") and would probe the ground with the palpi or even with the mandibulae. In absence of the leaf the dance stops after about a quarter of a minute and the beetle continues walking in the same direction as before (JERMY, 1961c).

The "dance" reflects a basic change in the coordinating centres because in this state the stimuli, represented by the surface on which the beetle walked, elicit probing responses which did not occur before. This state, therefore, can be regarded as a true CES.

The existence of an opposite state of the coordinating centres can be shown by the following:

The feeding of chewing insects consists of more or less distinct meals with shorter or longer periods of rest between them. During these periods the insect

remains on the place where it had fed or moves to a close point of the leaf. If, e.g. in the case of a feeding Colorado potato beetle larva, a drop of water is placed by a microsyringe on the surface of the leaf close to the mouth of the larva so that after a few bites the larva reaches the drop, no significant reaction can be observed. However, if instead of water the juice of a rejective non-host plant or the solution of an antifeedant is used, the larva stops feeding, and in the overwhelming majority of cases it does not remain on the same spot where it has been exposed to the inhibitory stimulus but moves away. Feeding recommences only after a relatively long pause. If the inhibitory stimulus is given again just after the larva recommences feeding, the same reaction can be observed as before.

Carrying out similar tests on adults of the Colorado potato beetle, the results differ only in the more intensive locomotor activity of the beetles after the exposure to the inhibitory stimulus.

Thus, the inhibitory stimulus induces a state of the central nervous system differing from its state when the animal is simply starving, since despite of starvation it does not respond now to the feeding stimuli present. Therefore, we can conclude that with regard to feeding this is a true central nervous system inhibitory state (CIS), (DETHIER, SOLOMON and TURNER, 1968).

The CIS has a decay time which lasts from the occurrence of the inhibitory stimulation to the recommencement of feeding. It is generally much longer than the decay time of the CES. In the 4th instar Colorado potato beetle larvae its length varies between a few minutes and about half an hour. The duration of the CIS depends on the degree of starvation ("motivation") and on some other unknown individual differences in the larvae.

Both the CES and the CIS are different from the state in which the central nervous system of the non satiated insect is during feeding or searching for food. Therefore, the existence of an intermediary state between the above-mentioned two states must be assumed. This can be defined as the basic or neutral state (CBS) of the coordinating centres.

It has to be emphasized that this relationship applies only to starving or non satiated insects. It has to be cleared whether satiation, which can be regarded as an "inner inhibition" of feeding, means a state different from the CIS or is identical with it?

The scheme in Fig. 1 shows the relationships between the stimuli, the states of the coordinating centres and the behavioural responses in the case of a starving insect.

The relation between inhibitory stimuli, CIS as well as duration and intensity of locomotion connected with the CIS varies considerably from species to species. This is reflected in the constancy of the physical contact between an insect and its host plant. From that point of view two groups of insects can be distinguished:

Group 1 includes insects which leave their food plants and return to them repeatedly in nature. They can be called as *dispersive forms*. Adults of *Coleoptera*

overwintering in places far from their food plants, larvae which feed during night and return to the soil in the morning (cutworms), and insects of similar behaviour belong to this group.

Group 2 consists of forms which usually do not leave their food plants during their whole life. These insects show restricted locomotor activity, thus, they can be regarded as *non-dispersive forms*. Larvae of most *Coleoptera*, *Lepidoptera*, *Tenthredinidae*, etc. are of that type.

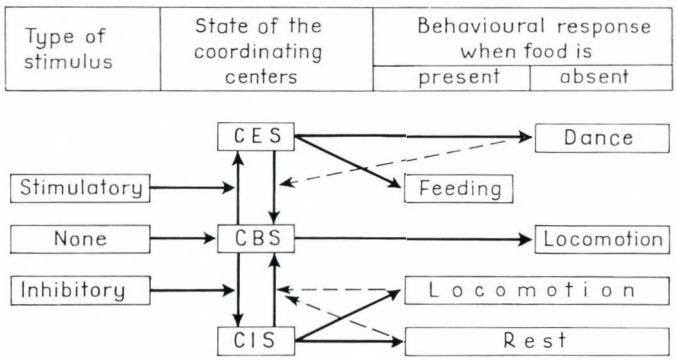

Fig. 1. Scheme showing the relations between the stimuli affecting food intake, the states of coordinating nerve centers, and the behaviour in a starving phytophagous insect. (CES = excitatory state, CBS = basic state, CIS = inhibitory state of the coordinating centres)

The two groups show different behaviour patterns related to the CIS. In the first group the CIS elicits intensive and long-lasting locomotion which makes the insect to leave the plant on which inhibitory stimuli acted. The adaptive value of this behaviour can be understood if one considers that in nature the insects find their food plants mostly by trial and error since no olfactory or visual clues lead them to the food from a greater distance (JERMY, 1961c). Thus, in most cases the insect searching for food meets and probes a series of non-host plants before it gets to its specific food. It has been also shown that the overwhelming majority of non-hosts plants contain feeding inhibitors (JERMY, 1958, 1966), thus, the probing of them elicits CIS which turns on intensive locomotion activity resulting in leaving the unsuitable plant within a short time. This mechanism increases the probability of finding a host plant after a minimum of trials.

If the host plant of a dispersive insect is treated with an antifeedant, the insect would leave it after a comparatively short time because of the intensive locomotor activity induced by the repeated inhibitory stimuli to which the insect is exposed when it probes the treated plant. Such insects would wander away from the treated plant very soon even if the antifeedant does not cover very evenly the surface of it.

However, the forms of the non-dispersive type, which are not adapted to

find and select their food in nature since they hatch on it, are behaviourally much stronger attached to their host plant. The cause of this is that in such insects the CIS induces mostly resting and not very intensive locomotion probably due to the stronger arrestant effect of olfactory stimuli. Therefore, if the host plant of an insect belonging to the non-dispersive group is treated with an antifeedant, the insect leaves the plant much later, if at all, and, compared to a dispersive insect, it insists much stronger to find a place on the plant where the antifeedant is absent, and continues feeding there. Thus, in such cases the antifeedant should be very evenly distributed all over the surface of the plant to provide effective protection against the insect.

Naturally, the above-mentioned two groups of insects represent extremes between which all intermediates can be found showing different mode and intensity of locomotor activity induced by the CIS. Therefore, it can be concluded that the knowledge of the behaviour related to the CIS in a given phytophagous insect is a valuable information for studying the possibilities of using antifeedants for pest control purposes (JERMY, 1968).

The question of the adaptation to inhibitory stimuli. The development of resistance to poisons is one of the steady increasing problems of the use of conventional insecticides. Thus, there is good reason to ask what the probability is of the appearance of insect strains resistant to an antifeedant?

For answering that question it has to be taken into consideration that a basic change in the host range of phytophagous insects is a very rare event both in natural and in agricultural biotops (HUFFAKER, 1957). So the host specificity can be regarded as one of the most conservative characteristics of phytophagous species. This is the more peculiar since in nature the phytophagous insects are very often forced to try feeding on other plants than their hosts, e.g., forms of the dispersive group when searching for food, larvae living on scattered plants in a herbaceous vegetation and destroying their host plant before reaching full growth, etc. Thus, a selective pressure which should result in often changes of host specificity is always present in nature, however, it does not seem to have any effect, presumably because the change in host specificity is related to a series of correlated changes in the function of chemoreceptors and in the behavioural responses to chemosensory inputs. The probability of such correlated changes must be very low.

Since the host range of phytophagous insects is mainly determined by the presence of deterrents, it can be concluded that the adaptation to deterrents, i.e. the development of insect strains resistant to antifeedants, must be very unlikely.

The influence of feeding inhibition on the population dynamics. The long decay time of the CIS and the locomotion related to it result in the reduction of food intake per unit of time and in the disturbance of the normal feeding rhythm. As a consequence, the larval development slows down and the fecundity of adults decreases. Thus, if the plant is made unsuitable for continuous feeding of a given

insect by treatment with an antifeedant, then the population dynamics of the insect is strongly influenced. This has been proved in laboratory and in small scale field experiments (JERMY, 1961b) as well as by large scale investigations carried out in Switzerland by MURBACH and CORBAZ (1963) who found that the mere deterring effect of copper compounds used against the potato blight decisively reduced the multiplication rate, and consequently the noxiousness of the Colorado potato beetle. When instead of copper compounds systemic fungicides with no deterrent effect were used, the damage caused by the Colorado potato beetle inereased again.

Chemical structure and deterrent effect. An important question arising in relation to the use of antifeedants is, what kind of substances are suitable for this purpose? Since in the case of oligophagous insects the chemoreceptors involved in controlling of feeding are sensitive to very different compounds (secondary and other plant substances) occurring in a huge number of their non-host plants and acting as deterrents, it can be *a priori* supposed that a very great variety of substances can play the role of antifeedants for a given insect species. Experiments carried out so far proved the validity of this assumption (Jermy, 1961a, 1966; WRIGHT, 1963; ASCHER, 1969).

On the other hand, it is also evident that two phytophagous species can react very differently to the same compound since substances present in the host plant of one species can be deterrent for the other and vice versa. Therefore, it is unlikely to find general antifeedants which would be equally effective against all or at least against a great number of insect species (JERMY and MATOLCSY, 1967). Copper ion seems to be one of the exceptions showing practically general antifeeding effect, but in this case the interaction between the ion and the receptor cells might be different from that with organic compounds.

The fact that substances of very different chemical structure can inhibit feeding of a given insect species is very advantageous because it enables to find substances most suitable for practical purposes.

Problems of the Practical Use of Antifeedants

The most important requirements which should be met by an antifeedant for the use in plant protection are the following:

(1) Strong antifeedant action so that relatively small quantities per unit of area have to be used.

(2) Adequate persistence so that the treated plant is protected for a time span determined by biological and economical aspects.

(3) Systemic action assuring the translocation of the substance into the non-treated parts of the plant and into the young parts developing after treatment.

(4) Lack of any harmful effects on the plant, on animals (including natural enemies of the pest, pollinators, etc.) and first of all, on man.

(5) Economic feasibility.

Until now the suitability of copper, organotin and some triazene compounds for field application has been proved. (See the review by ASCHER, 1969). However, these compounds do not fulfil all requirements mentioned above, and none of them is systemic. Therefore, the author and his co-workers began to study various substances known to be systemic. Among them various derivatives of phenoxyacetic acid and phenoxyethanol showed strong antifeedant effect against several chewing and sucking insects, and phenoxyethanol against the red spider mite, *Metatetranychus ulmi* Koch (JENSER, 1967; JERMY and MATOLCSY, 1967; MATOLCSY et al., 1968).

Despite of the encouraging results got in the laboratory, the field trials carried out so far with the new compounds have not been conclusive enough. It seems that there is still a long way to go before an antifeedant can be introduced into practice. However, the use of feeding inhibitors is biologically well founded and principally very promising so that it must be included into the series of new approaches to more selective methods of insect pest control.

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Toxicological, Chemosterilant and Histopathological Effects of Triphenyl-tin-hydroxide on *Spodoptera littoralis* Boisd.

By

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Laboratory and field experiments were conducted in the United Arab Republic to study the toxicological, histopathological and chemosterilizing effects of Du-Ter (triphenyl-tin-hydroxide) on the cotton leafworm, *Spodoptera littoralis* Boisd. The results showed that Du-Ter had a higher effectivity on the cotton leafworm when used as a stomach poison, compared to topical applications on larvae. There was also a pronounced antifeeding effect of the preparation on orally treated larvae. Du-Ter had a sterilizing effect on both sexes, when used on larval food in sublethal doses; the effect was more severe on males than on females. Both egg production and egg viability were severely affected when Du-Ter was used orally or topically on adults. The preparation was found to cause pronounced effects on various larval tissues, resulting in partial destruction of mid-gut, muscles, fat bodies and Malpighian tubules. These properties seem to be in connection with its action as stomach poison.

Insecticides of different groups are used extensively every year for the control of the cotton leafworm *Spodoptera littoralis* Boisd. in the U.A.R. However such heavy applications resulted in serious problems and it was felt necessary to explore new methods.

Among the new approaches used by different workers in the U.A.R. for tackling the cotton leafworm problem was the application of chemosterilants and antifeedants.

Prelimiray studies obtained by Henien (1968) indicated that the two antifeedants Brestan and Du-Ter could be used successfully for the control of the cotton leafworm. However the work of Henien left many questions to be answered such as: the mode of action of these materials, the migration of the cotton leafworm from treated to untreated plants and the histopathological effects of such compounds.

The present work has been conducted to cover the previously mentioned lackings, and Du-Ter was chosen for this purpose. Beside its antifeeding studies, the chemosterilant action of Du-Ter against the cotton leafworm has been also investigated.

I. Toxicological Studies

The aim of the present work was to study the effect of Du-Ter toxicologically on cotton leafworm *Spodoptera littoralis* BOISD. For this reason series of experiments were carried out in the following part.

The different stages of the cotton leafworm *Spodoptera littoralis* BOISD. needed for the present work were obtained from laboratory colony reared under standard conditions at the Entomology Department, Faculty of Agriculture, Kafr El-Sheikh. The stock colony was reared on Caster bean leaves according to the method of ZAKI et al. (1966).

Du-Ter was the only tested material. It was used as active ingredient (triphenyl-tin-hydroxide) as a wettable powder formulation containing 50% active ingredient.

Study of contact and stomach action

Contact action: In this experiment the topical application technique was used by means of a microsyringe. A series of dosages of the active ingredient of Du-Ter dissolved in acetone was prepared as follows: 1000, 800, 600, 400, 200, and 100 p.p.m. The above mentioned dosages were applied to the dorsal surface of the third thorathic segment of the larva.

Stomach action: In this technique the material was administered directly to the stomach through the buccal cavity using a microsyringe furnished with curved needle. Precautions were taken to avoid contamination of the mouth parts with Du-Ter. The active material was applied dissolved in acetone at the same dosages mentioned before.

In both techniques the third larval instar of cotton leafworm was used as an experimental insect. 100 larvae were used for each dosage in both methods of application. Mortalities were recorded daily for a period of three days after treatment.

Treated larvae were classified into two categories:

1) larvae, which failed completely to feed.

2) larvae with reduced feeding capacity.

In the latter case the capacity of feeding has been estimated in the terms of the percentage of food consumption. Larvae treated with acetone alone were used for both experiments as check.

Results and discussion: It is evident from data in Table 1 that Du-Ter was much stronger as a stomach poison than as a contact poison. Such phenomenon was observed for all dosages and in all intervals.

In Tables 2 and 3 we summarized the number of nonfeeding and partially feeding larvae when treated by both contact and stomach techniques. The antifeeding effect of Du-Ter when used orally is shown in Table 2. Results indicate that the percentage of nonfed larvae increased as the dosage was increased.

Table 1

Daily recorded mortality obtained from contact and stomach action of different concentrations of Du-Ter
(A. I. in acetone)

Dose/larva (p.p.m.)	Mortality %					
	Stomach action			Contact action		
	24 hr.	48 hr.	72 hr.	24 hr.	48 hr.	72 hr.
100	10	20	52	0	0	8
200	15	25	48	0	4	14
400	25	50	80	4	12	26
600	68	70	88	8	18	56
800	75	100	—	6	24	64
1000	82	100	—	8	36	72
Acetone alone	0	0	0	0	0	0
Check	0	0	0	0	0	0

Table 2

The antifeeding effect of Du-Ter (A. I. in acetone) through the stomach action

Dose/larva (p.p.m.)	Number of nonfeeding larvae								
	1*st* day			2*nd* day			3*rd* day		
	Survivals	Nonfed.	%	Survivals	Nonfed.	%	Survivals	Nonfed.	%
100	100	45	45	90	55	61.1	80	30	37.5
200	100	82	82	85	75	88.2	75	67	89.3
400	100	92	92	75	55	73.3	50	45	90.0
600	100	95	95	32	32	100.0	30	30	100.0
800	100	95	95	25	25	100.0	0	—	—
1000	100	95	95	18	18	100.0	0	—	—
Acetone alone	100	3	3	100	2	2.0	100	00	00.0
Check	100	00	00	100	00	0.0	100	00	00.0

It was observed also that the percentage of nonfed larvae increased by time.

Concerning the antifeeding effect of Du-Ter when used by contact, its poor action was noticed, when compared with that of the stomach action (Table 3).

The Af.D_{50} values were obtained from the antifeeding regression lines, and given in Table 4. The expression Af.D_{50} in this work was applied to the dosage of Du-Ter causing the absolute nonfeeding of 50% of larvae after 3 days. The dosages required to obtain Af.D_{50} was 183 p.p.m. per larva of active ingredient

Table 3

The antifeeding effect of Du-Ter (A. I. in acetone) through the contact action

Dose/larva (pp..m.)	Number of nonfeeding larvae								
	1*st* day			2*nd* day			3*rd* day		
	Survivals	Non-fed.	%	Survivals	Non-fed.	%	Survivals	Non-fed.	%
100	100	00	00	100	12	12.0	100	00	00.0
200	100	12	12	100	18	18.0	96	00	00.0
400	100	16	16	96	18	18.7	88	00	00.0
600	100	18	18	92	24	26.0	82	12	14.6
800	100	24	24	94	40	42.5	76	16	21.0
1000	100	48	48	92	60	65.2	64	52	81.2
Acetone alone	100	00	00	100	00	00.0	100	00	00.0
Check	100	00	00	100	00	00.0	100	00	00.0

Table 4

The Af. D_{50}'s values obtained from contact and stomach action of Du-Ter when applied as active ingredient to the 3*rd* instar of the cotton leaf-worm during the three days of experiment

Dose/larvae (p.p.m.)	No. of absolutely non-feeding larvae		Af. D_{50} in p.p.m.	
	Stomach	Contact	Stomach	Contact
100	20	0		
200	76	6		
400	76	12		
600	95	22	183	2158
800	95	26		
1000	95	48		
Acetone alone	00	00		
Check	00	00		

when the material was used orally, while it reached 2158 p.p.m. when it was used by contact method. Therefore, it could be concluded that the antifeeding action of the material was highly effective when it was applied orally, while the contact treatment was less effective.

Starvation studies

Methods and techniques. This experiment was conducted to study the effect of both natural and artificial starvation on the larva of cotton leafworm *S. littoralis* BOISD.

Four groups, 20 each of 4th larval instar of cotton leafworm were starved naturally at the intervals of 6, 24, 48 and 96 hours. Larvae of similar age (20 in No.) were used without starvation as check. A group of 20 of 4th instar of nonstarved larvae were fed on leaves previously dipped in a concentration of 0.25% of antifeeding agent (Du-Ter W.P. 50%). The leaves were allowed to dry before using them.

Caster bean leaves were used as food during the experiment. The leaves were selected of standard size and without holes or punctures. Each larva was reared separately in a rearing vial. Each vial at natural starvation was provided

Table 5

Daily mortalities for natural and induced starvation

Treatments	Mortality % at day											$L.T_{90}$ by days	$L.T_{50}$ days
	1	2	3	4	5	6	7	8	9	10	11		
Natural starvation for 6 hrs.	00	00	00	00	00	00	00	00	00	10	P	—	—
24 hrs.	00	00	00	00	00	00	10	10	00	P		—	
48 hrs.	00	00	00	00	00	10	20	50	P			8.15	10.40
96 hrs.	20	30	50	50	60	80	P					3.55	7.65
Induced starvation (Du-Ter treat.)	00	10	40	70	90	100						3.30	5.05
Check	00	00	00	00	00	00	00	00	00	00	P	—	—

Induced = Du-Ter treatment
P = Pupated

daily after starvation period with a new fresh caster bean leaf, while vials at induced starvation were provided with one treated leaf during the first 48 hours, then the treated leaf was replaced daily with a new fresh clean leaf till pupation (P.) or death.

Results and discussions. Results obtained in Table 5 indicate that the mortality caused by natural starvation increased by time increase.

However, much higher mortality was obtained through induced starvation. Natural starvation for 96 hours caused 80% mortality compared with 100% mortality for induced starvation.

It could be noticed also that induced starvation caused higher mortality in a shorter time than natural starvation, where the $L.T_{90}$ values were 10.4, 7.65 and 5.05 days for natural starvation of 48 hrs, 96 hrs and Du-Ter treatment respectively. This confirms the idea that the effect of Du-Ter could not be due to starvation only.

The high mortality obtained from induced starvation (Du-Ter treatment) could be explained on the basis that the antifeeding action of Du-Ter was coupled with its strong stomach action.

Feeding response of larvae to different host plants treated with Du-Ter

Methods and technique. This experiment was carried out to investigate the response of the cotton leafworm larvae to different host plants treated with

Table 6

Percentages of consumed area in different host plants treated with Du-Ter W. P. 50% after 24 and 48 hours

Host plant	Average consumed area/larva (cm^2)			
	Untreated leaves (check)		Treated leaves (Du-Ter)	
	24 hrs	48 hrs	24 hrs	48 hrs
Castor bean	8.57	9.75	0.92	1.00
Cabbage	6.30	8.67	0.67	1.29
Cotton	7.44	8.58	0.50	0.52
Sweet potato	6.50	8.25	2.52	2.77
Grape	4.85	7.50	1.60	1.67
Average	6.73	8.55	1.24	1.45
Tomato	2.62	6.35	0.25	0.25
Snap bean	3.47	4.34	0.35	0.42
Maize	3.40	4.00	0.12	0.12
Potato	2.78	3.69	0.23	0.27
Egg plant	2.60	3.58	0.13	0.17
Average	2.97	4.39	0.21	0.24

Du-Ter. Ten preferred host plants were chosen as food to the cotton leafworm, For this purpose the leaves of the following plants were used: tomatoes, maize, castor beans, cotton, potatoes, grapes, snap beans, egg plant, cabbage and sweet potatoes. Du-Ter was applied as a wettable powder formulation containing 50% using the hand sprayer atomizer.

Two groups of leaves from each host plant were prepared without any feeding punctures. Each group contained 100 leaves, nearly equal in size. The first group of leaves was sprayed with Du-Ter at the concentration of 0.25% and were left to dry. One 4th instar larva was introduced to each leaf, and then placed in a rearing glass vial. The second group of leaves was used without treat-

ment as check. Larvae were transferred to leaves, one larva per leaf separately in a rearing glass vial.

The petioles of the leaves were surrounded with small pieces of wet cotton to keep the leaves fresh as long as possible. Results were recorded at 24 and 48 hours. The average of consumed area and mortality percentages were recorded.

Results and discussion. Generally it would be noticed from data obtained in Table 6 that the host plants used could be differentiated into two distinct groups. The first group was considered preferable to the cotton leafworm and includes: caster bean, cabbage, cotton, sweet potatoes and grape. The second group was considered less preferable to the cotton leafworm and includes: tomatoes, snap bean, maize, potatoes and egg plant.

According to the feeding data in Table 6 the average untreated consumed area in the first group was 8.55 cm^2 compared with 4.39 cm^2 in the second group. With treated leaves the same trend could be observed, where the average consumed area in the first group was 1.45 cm^2 compared with 0.24 cm^2 in the second group. Therefore it could be concluded that the feeding response of the cotton leafworm depends mainly on the host plant preference whether treated with Du-Ter or not.

II. Chemosterilant Studies

Reviewing the literature revealed the importance of tin derivatives as reproduction inhibitors.

KENAGA (1965) reported that numerous triphenyl tin compounds inhibited the reproduction in the house fly.

WOLFENBARGER et al. (1968) found that less than 2% of the eggs laid by moth emerging from tobacco budworm larvae treated with acetoxytrimethyl tin and hydroxytriphenyl tin hatched.

SIDNEY (1968) studied the inhibition of reproduction in *Musca domestica* L. with triphenyl tin acetate and triphenyl tin chloride. When offered in the diet to ovipositing females both tested compounds reduced the number of eggs produced and per cent hatch.

Since Du-Ter is a tin derivative, so it was found that it would be more useful to explore the chemosterilant effect of such compound.

Methods and technique

1. *Larval treatment.* Du-Ter was tested as a chemosterilant against the third larval instar of *S. littoralis* BOISD. This stage was chosen purposely since it proved from the previous study to be the most tolerant to the action of Du-Ter. The indirect technique (spraying leaves only) was used also to represent the actual treatment in the field. A series of concentrations of Du-Ter W.P. 50% were used in water as follows: 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5 and 1.0%. Hundred

larvae were used for each concentration. Caster been leaves were dipped in the used concentration and left to dry then introduced to the larvae. One leaf was offered to each larva in a separate vial glass.

Larvae were left to feed on the treated leaves for 48 hours, then survived larvae were provided daily with clean fresh leaf.

Mortality percentages during the larval stage were calculated daily till pupation. Also daily consumed area per larva was measured in square centimeters.

Percentages of pupal survivals were estimated. Consequently pupal mortality was determined after the emergence of moths took place. Moths obtained from each treatment were allowed to mate as follows:

1) Male produced from treated larva × female produced from rearing colony not contaminated with any chemicals.

2) Female produced from treated larva × male produced from rearing colony.

3) Male × female, both reared from treated larvae.

4) Males and females from rearing colony was check.

Each of the prementioned combinations was replicated 5 times for each treatment used. Each pair was confined to a single glass container, which was provided with a piece of cotton wool saturated with 10% sugar solution for moth feeding.

Oviposition was observed and eggs laid were recorded daily. Eggs were then transferred to a Petri dish and left to hatch. Hatched and nonhatched eggs were counted. Adult longevity as calculated.

2. *Adult treatment.* The direct chemosterilant action of Du-Ter on moths of the cotton leafworm was studied in two ways, orally and by contact.

Contact application. Two big jars were coated internally with Du-Ter W.P. 50% and each was provided with 5 cotton leaves sprayed with the same concentration. Untreated, newly emerged moths were transferred to the sprayed jars, each sex separately in one jar. Moths were allowed to stay for a period of 24 hours in these treated jar, after which they were transferred in pairs to small jar (1 lb) provided with a piece of cotton wool soaked in 10% sugar solution only. Each jar was provided also with fresh clean cotton leaf and was covered with muslin cloth and secured with rubber band. Confined moths were in pairs in the jars as follows:

a – Treated male × treated female.

b – Treated male × untreated female.

c – Untreated male × treated female.

d – Untreated male × untreated female.

Each of the above mentioned combinations was replicated 5 times.

Oral applications. In the oral application the required amount of Du-Ter W.P. 50% was dissolved in 10% sugar solution to obtain the concentration required. Newly emerged moths were transferred into two big jars provided with a

piece of cotton wool soaked in Du-Ter sugar solution. Sexes were kept separately. Untreated moths were placed in 2 jars, each provided with a piece of cotton wool soaked in 10% sugar solution only. Moths were allowed to feed in both cases for 24 hours then were paired in small vials (1 lb) as previously mentioned in contact application. Each treatment was replicated 5 times for each concentration. Each vial was provided with one fresh cotton leaf and a piece of cotton wool soaked in 10% sugar solution, then was covered with cheesecloth and secured with rubber band.

Table 7

The lethal and sublethal effect of Du-Ter W. P. 50% on cotton leafworm larvae at different concentrations when used sprayed on leaves (indirectly)

Concentration %	Mortality percentage at day											LT_{50} by days
	1	2	3	4	5	6	7	8	9	10	Total	
0.5000	54	90	100								100	0.95
0.4000	32	86	100								100	1.30
0.3000	26	80	98	100							100	1.50
0.2000	8	76	98	100							100	1.70
0.1000	4	69	96	96	96	98	98	100			100	1.90
0.0500	2	42	86	88	90	90	92	94	94		94	2.25
0.0100	0	16	18	34	48	50	62	72	80		80	5.80
0.0050	0	4	16	24	32	46	54	60	66	68	68	6.80
0.0010	0	2	14	16	21	32	46	54	60	66	66	7.85
0.0005	0	0	2	6	13	22	35	51	59	61	61	8.10
Check	0	0	3	5	9	9	23	28	37	47	47	

LT_{50} = Time at which mortality reaches 50%

For both contact and oral applications the concentrations used were 0.1, 0.3, and 0.5% of Du-Ter W.P. 50%.

Daily observations were made to record the number of egg masses laid. These were transferred daily to small vials until hatching took place. Percentage of hatchability was recorded. Records for adults longevity were obtained for both sexes at the end of experiment.

Cotton leaves in each jar were removed daily and replaced with fresh leaves.

Results and discussion

The chemosterilant action of Du-Ter on the 3rd larval instar of cotton leafworm. It was clear from the data in Table 7 that concentrations exceeding 0.1% were completely lethal to larvae, while concentrations below this level were of sublethal effect.

The LT_{50} proved to have positive correlation with different concentrations used; where it was 0.95, 2.25, 6.8, and 8.1 days at concentrations of 0.5, 0.05, 0.005, and 0.0005 respectively.

It was evident from Table 8 that the number of pupae obtained increased with the decrease of concentrations. For example pupal formation reached 6, 20, 32, 34 and 39% for 0.05, 0.01, 0.005, 0.001 and 0.0005% concentrations compared to 53 pupae formed at the control.

Table 8

The effect of Du-Ter W. P. 50% when used at larval feeding period on pupation and pupal viability

Concentration	Total No. of		No. of dead pupae during pupal stage			Pupal stage mortality %	Pupal mean weight by gm.
	larvae used	pupa formed	At beginning	At end	Total		
0.5000	100	—	—	—	—	—	—
0.4000	100	—	—	—	—	—	—
0.3000	100	—	—	—	—	—	—
0.2000	100	—	—	—	—	—	—
0.1000	100	—	—	—	—	—	—
0.0500	100	6	2	3	5	83.3	0.187
0.0100	100	20	2	12	14	70.0	0.183
0.0050	100	32	2	13	15	46.9	0.189
0.0010	100	34	2	16	18	46 1	0.222
0.0005	100	39	3	7	10	25.6	0.249
Check	100	53	2	2	4	7.5	0.287

N. B.: Pupae obtained from 0.05 and 0.01% concentration were not enough for completing the following tests, therefore it were repeated 3 times

The vitality of pupae formed, was adversely affected by the concentration sprayed on leaves fed to larvae. Percentages of pupal mortality amounted to 70, 46.9, 46.1 and 25.6% at concentrations of 0.01, 0.005, 0.001, and 0.0005% respectively.

Records presented in Table 9 clearly indicate that egg production was proportionally reduced with the increase of Du-Ter. Reduction in fecundity was much pronounced when treated males were mated with untreated females than vice versa. This indicates that the male is more sensitive to Du-Ter than the female. Reduction percentages in fecundity reached 24.5, 35.0, 64.7, 71.7, and 76.1% for treated males compared with 10.2, 23.8, 57.0, 69.4 and 76.5% for treated females; at concentrations 0.0005, 0.001, 0.005, 0.01 and 0.05% respectively.

On the other hand, when both sexes were treated as larvae and were mated, fewer eggs were laid; reduction in total number of eggs laid were 81.9, 74.1,

Table 9

The effect of Du-Ter W. P. 50% when used at larval feeding period on fecundity of adults obtained

Concentration % used for larvae	Average No. of eggs laid/pair						Mean No. of egg/pair for concentration
	♀♂ T		T ♀♂		T ♀♂ T		
	Laid	% Reduction than control	Laid	% Reduction than control	Laid	% Reduction than control	
0.0500	314	76.1	309	76.5	238	81.9	287
0.0100	372	71.7	402	64.4	341	74.1	272
0.0050	469	64.7	566	57.0	345	73.8	458
0.0010	856	35.0	1003	23.8	769	41.6	876
0.0005	994	24.5	1182	10.2	1187	9.8	1121
Check							1317

T = Moth obtained from 3*rd* larval instar feeding on treated leaves

Table 10

The effect of Du-Ter W. P. 50% when used at larval feeding period on egg viability and adults obtained sterility

Concentration % used for larvae	Sex combination used						Mean/concentration	
	♀♂ T		T ♀♂		T ♀♂ T			
	Viability %	Stertility %	Viability %	Sterility %	Viability %	Sterility %	Viability %	Sterility %
0.0500	0	100.0	37.0	63.0	16.0	84.0	17.7	82.3
0.0100	10.8	89.2	43.0	57.0	16.0	83.4	23.5	76.5
0.0050	18.9	81.1	45.8	54.2	26.1	73.9	30.3	69.7
0.0010	21.1	78.9	61.7	38.3	36.0	64.0	39.6	60.4
0.0005	44.7	55.3	86.2	13.8	52.0	48.0	61.0	39.0
Check	100.0	0					100.0	0

T = Moth obtained from 3*rd* larval instar feeding on treated leaves

73.8 and 41.6 per cent, using concentrations of 0.05, 0.01, 0.005, and 0.001 respectively.

It is clearly observed from data in Table 10 that Du-Ter, when applied to larval food had a sterilizing effect, where it affected the viability of eggs laid by obtained moths. The percentages of egg hatchability produced by treated female and male moths were 52.0, 36.0, 26.1, 16.6, and 16.0% at concentrations 0.0005, 0.001, 0.005, 0.01 and 0.05% respcetively.

Results on sex sterilization proved that the egg viability was affected according to the treated sex and that males were more severely affected than females.

Percentages of sterility were 55.3, 78.9, 81.1, 89.2 and 100% for treated males mated with untreated females compared with 13.8, 38.3, 54.2, 57, and 63% sterility for treated females mated with untreated males at 0.0005, 0.001, 0.005, 0.01, and 0.05% concentrations of Du-Ter W.P. 50.%

The chemosterilant effect of Du-Ter on adult moths of cotton leafworm:

a) *Oral effect on both sexes separately and combined.* Data shown in Table 11 clearly indicated that both sexes were affected in two different ways when were allowed to feed on Du-Ter sugar solution.

The first effect was shown in the form of reduction in number of eggs laid. Secondly egg viability was severely influenced.

A positive correlation was recorded between the concentration used and the percentage of reduction in fecundity, meaning that the higher the concentration used, the higher the percentage of reduction obtained. For instance, when both sexes were treated, 96.1% reduction was associated with 0.5% concentration while 72% reduction was obtained with 0.1% concentration.

The effect of the material on the egg viability was so distinct at higher concentrations of 0.5 and 0.3% that no eggs hatched at such concentrations; while the lower concentration of 0.1% allowed 49.9% egg viability when both sexes were treated.

b) *Contact effect on both sexes separately and combined.* The contact effect of Du-Ter was studied by exposing moths to films of residues on the inner surface of glass jars and films on cotton plant leaves both together. Period of exposure to the residues lasted for 24 hours.

Results presented in Table 12 showed that egg production was decreased as the material concentration increased. It was also noticed that the male was more easily affected than the female where the percentages reduction in eggs laid reached 81.7, 83.4 and 91.2% when males were treated only, while 65.1, 74.7 and 79.0% when females were treated only at concentrations of 0.1, 0.3 and 0.5% respectively.

When evaluating the sterilizing action of Du-Ter, it could be observed clearly that the material gave a lower sterilizing action when used by contact. When both sexes were exposed to a residual film, percentages of egg sterility were 34.1, 37.5 and 66.1% using concentrations of 0.1, 0.3 and 0.5%. It was also observed that males were more affected than females.

Sterility data shown in Tables 11 and 12 were used for drawing sterility regression lines. $S.c_{50}$ and $S.c_{90}$ values as obtained from these regression lines were tabulated in Table 13.

Using the material orally $S.c_{50}$ were 0.128, 0.310 and 0.098% when using treated males, treated females and combination of both treated sexes respectively, while when using the material as residual films the $S.c_{50}$ were 0.43, 0.74 and 0.33% for treated male only, treated female only and combination of both treated sexes respectively.

Table 11

The oral effect of Du-Ter on fecundity and sterility of the cotton leaf-worm moths when it were fed for 24 hours on 10% sugar solution containing different concentrations of Du-Ter W. P. 50%

Concentration used orally %	Sex combination	Average No. of eggs laid/pair	Average No. of hatched eggs	Reduction % than control	% Viability	% Sterility
	♀ ♂ T	561	350	67.4	62.3	37.7
0.1	T ♀ ♂	482	348	72.0	72.2	27.8
	T ♀ ♂ T	477	239	72.3	49.9	50.1
	♀ ♂ T	489	17	71.6	3.5	96.5
0.3	T ♀ ♂	324	209	81.2	64.5	35.5
	T ♀ ♂ T	326	0	81.1	0	100.0
	♀ ♂ T	327	0	81.0	0	100.0
0.5	T ♀ ♂	258	82	85.0	31.8	68.2
	T ♀ ♂ T	67	0	96.1	0	100.0
Control		1725	1725	—	100.0	0

Table 12

The contact effect of Du-Ter on fecundity and sterility of cotton leafworm moths when it were exposed for 24 hours to residual films of different concentrations of Du-Ter W. P. 50%

Concentrations used as contact %	Sex combination	Average No. of eggs laid/pair	Average No. of hatched eggs	Reduction % than control	% Viability	% Sterility
0.1	♀ ♂ T	315	241	81.7	76.5	23.5
	T ♀ ♂	602	492	65.1	81.7	18.3
	T ♀ ♂ T	305	201	82.3	65.9	34.1
0.3	♀ ♂ T	285	196	83.4	68.8	31.2
	T ♀ ♂	535	404	74.7	75.5	24.5
	T ♀ ♂ T	267	167	84.5	62.5	37.5
0.5	♀ ♂ T	152	56	91.2	36.8	63.2
	T ♀ ♂	361	214	79.0	59.3	40.7
	T ♀ ♂ T	212	72	87.7	33.9	66.1
Control		1725	1725	—	100.0	—

T = Treated as imagines

From data obtained in Table 13 it could be seen that Du-Ter was more effective when applied to both sexes than when used to one sex only. However, it was more effective against males than females, where the relative potency was 5.78 and 2.39 respectively when used orally.

Table 13

S. C. values and relative potencies of Du-Ter W. P. 50% when used orally and residual films on moths of the cotton leafworm

Treated sex	$S.C._{50}$		$S.C._{90}$		Relative potency (according to $S.C._{50}$)	
	Orally	Contact	Orally	Contact	Orally	Contact
T ♀ ♂	0.310	0.74	2.80	12.5	2.39	1.00
♀ ♂ T	0.128	0.43	0.28	5.0	5.78	1.72
T ♀ ♂ T	0.098	0.33	0.20	7.0	7.55	2.24

T = Treated as moths
$S.C_{50}$ = The concentration which caused 50% sterility
$S.C_{90}$ = The concentration which caused 90% sterility

III. Histopathological Studies

Results obtained from the toxicological studies indicated clearly that Du-Ter proved to be more effective as a stomach poison than it did as contact poison. For this reason it has been felt that histopathological studies might be useful to throw light on the site of action of Du-Ter.

Therefore the following studies were conducted chiefly to investigate histopathologically the effect of Du-Ter on the larvae of cotton leafworm.

Methods and techniques

Fourth instar larvae of the cotton leafworm were used as testing insect. It was fed on castor oil leaves sprayed with a concentration of 0.25% of Du-Ter 50% W.P.

Treated worms were picked up for histological studies after forty eight hours before the post mortem symptoms interfere. The period of 48 hours was determined from the previous mortality experiments.

To study the changes in the different tissues caused by the antifeeding material, it was necessary to study the normal histology of the different tissues of untreated larvae, therefore healthy worms of the same age were picked up at the same time and were dissected.

Both kinds of larvae are fixed in warm Bouin's solution (at 60°C) where they are left for two minutes, then they were transferred to cold Bouin and left for 24 hours. The material is then washed in 70% ethanol alcohol and transferred to 80% ethanol and left there for 24 hours and then follow the next transfers: 90% ethanol for 4 hours, 95% ethanol for 2 hours, 100% ethanol for three hours (3 changes) and then clove oil overnight.

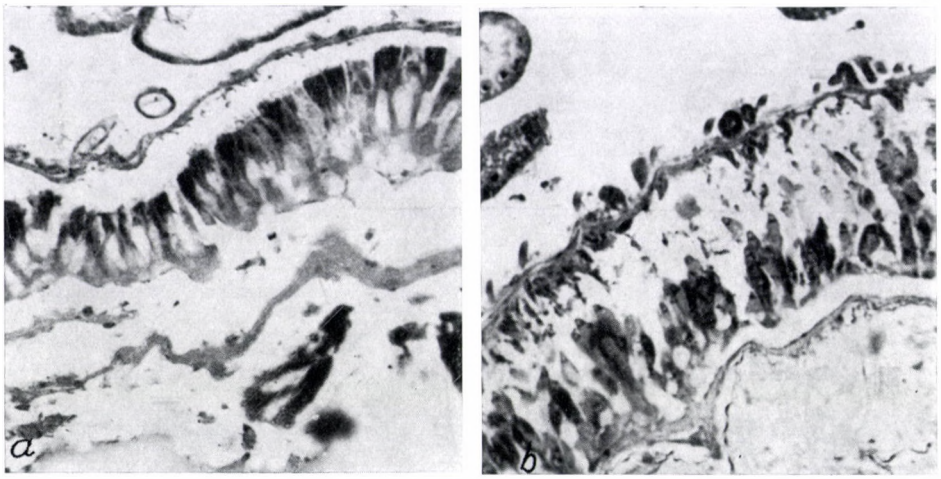

Fig. 1. Part of a transversal section in mid-gut of cotton leafworm. Left: healthy larva; right: Du-Ter treated larva

The clove oil is removed from the material by washing in xylene for 15 minutes, then it passed through xylol-paraffin (1 : 2) for 15 minutes, paraffin wax for 3 hours (3 changes; ½, 1, 1 ½ hours). The materials were then embedded in paraffin wax and prepared for sectioning. Sections are cut at a thickness of about 6 microns. Delafield's haematoxylin and eosin were found satisfactory in staining for the purpose of the present study.

The tissues which were under investigation were the midgut, fat bodies, Malpighian tubules, and the muscles. A description of the above mentioned tissues in healthy larvae and the histopathological changes in treated larvae, due to Du-Ter are given in the following and was represented with photomicrographs for the cross-sections in it.

Results and discussion

The histology of diseased tissues was described to indicate the effect of Du-Ter on various tissues of cotton leafworm larvae.

Effect on mid-gut (Fig 1): The musculosa of the mid-gut of treated larvae was slightly detached from the epithelial layer. In some parts, the longitudinal muscles were much larger than the ones of healthy larvae. The basement membrane was destroyed and the regenerative cells were severely destructed and distributed in the space formed between musculosa and epithelial layer. The nuclei of epithelial cells were elongated and taking spinal form with granular particles filling up the space of the cell. The calyciform cells were destroyed leaving many spaces throughout the epithelial layer. The inner surface of the epithelial cells was characterized by the presence of particles of different sizes.

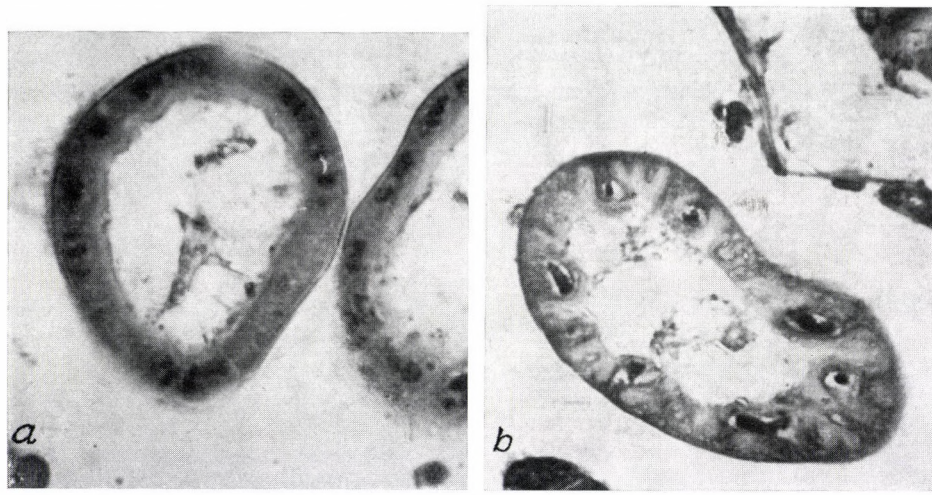

Fig. 2. Transversal section in Malpighian tubules of cotton leafworm. Left: healthy larva; right: Du-Ter treated larva

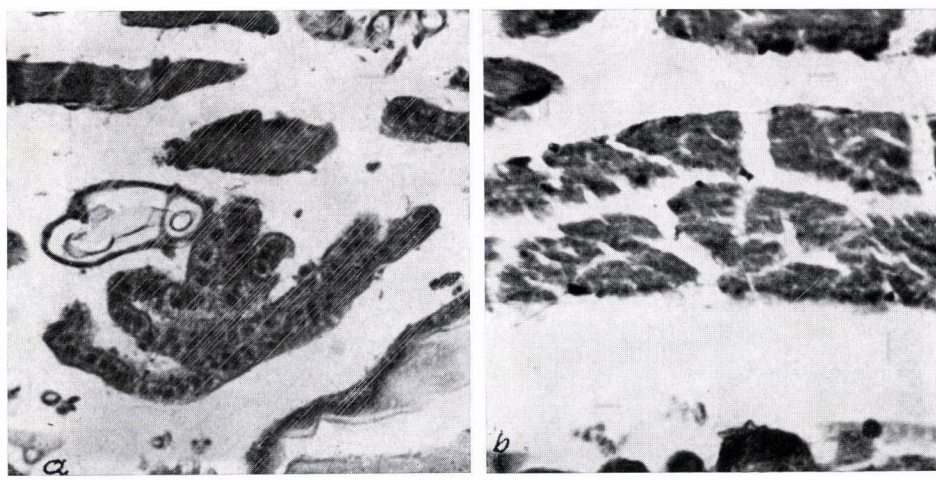

Fig. 3. Section in fat tissue of cotton leafworm. Left: Healthy larva; right: Du-Ter treated larva

The peritrophic membrane was clear as boundary for the lumen of mid-gut. Between the peritrophic membrane and the particles of the epithelial cells, there was a clear layer of uncellular materials distributed in many sites.

Effect on Malpighian tubules (Fig 2). No changes took place in the peritoneal and basement membranes. However nuclei were affected, chromatin substances

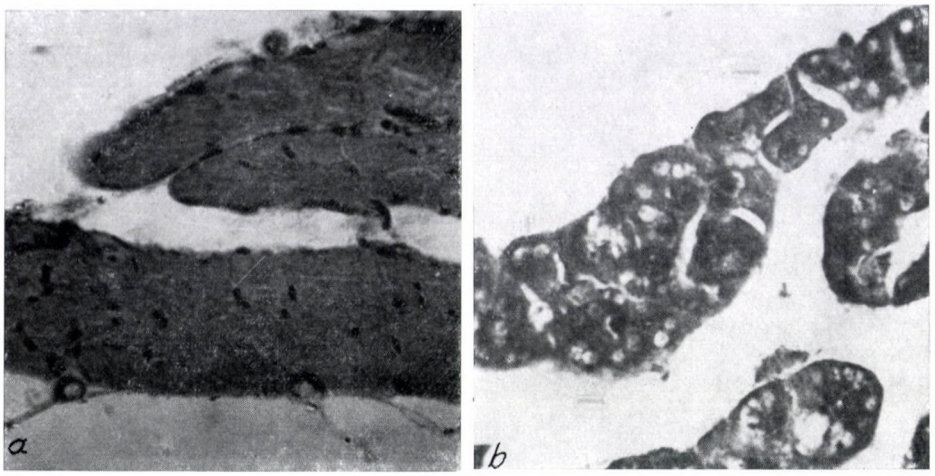

Fig. 4. Section in muscles of cotton leafworm. Left: Healthy larva; right: Du-Ter treated larva

were seen accumulated in a circular mass leaving a free area just inside the nuclear membrane. In addition the epithelial cells showed considerable variations in size. Many degenerated parts were found falling in the lumen.

Effect on fat bodies (Fig. 3). The membranous sheath of the fat body ribbon was destroyed in some parts and the fat cells seemed to be disclosed together in the ribbon. The cytoplasm of the affected fat cells was also shrunken due to the presense of the vacuoles. The nuclei appeared to be slightly degenerated.

Effect on muscles (Fig. 4). Destruction of the muscle bundle took place in all directions forming irregular masses of myofibrils.

The sarcolemma was partially destroyed and the muscle fibres were broken into several parts.

Toxicological studies carried out in the present work led to the following conclusions:

IV. General Conclusions

1. *Toxicological studies*

Few workers have carried out studies on the effect of antifeedants on the cotton leafworm, due to the recent discovery of such compounds.

BULLOCK et al. (1963) studied the antifeeding effect of DDT on bollworms and tobacco budworm larvae. He found that both species gave an anti-feeding response of a similar degree to DDT. HENEIN (1968) concluded that the death of the cotton leafworm fed on leaves treated with Du-Ter and Brestan was mainly due to starvation.

Contact and stomach action. Data obtained in this connection proved quite clearly that Du-Ter was much stronger as a stomach poison than as contact poison. However in both methods of applications, there was a positive correlation between the dosage and mortality. Using Du-Ter orally resulted, in $L.D_{50}$ value of 297 p.p.m. active ingredient while it was 2135 p.p.m. by contact after 48 hours.

Starvation studies. In a comparative study between the starvation due to the effect of Du-Ter and natural starvation, results indicated that Du-Ter caused higher mortality (100%) in a shorter time than natural starvation (80%). Such fact indicates that starvation is not the only factor causing the death of cotton leafworm when fed on leaves treated with Du-Ter, and that confirms the toxic action of Du-Ter either by stomach or by contact.

The antifeeding action of Du-Ter could be considered as a subsequent result of the strong stomach action of the compound. Such a conclusion has been drawn from the positive correlation between the dosage and mortality and from the negative correlation between the dosage and the feeding capacity.

The feeding response of larvae to different host plants treated with Du-Ter. According to feeding capacity evaluation, feeding response of cotton leafworm depends mainly on the host preference, whether plants were treated with Du-Ter or not.

2. *Chemisterilant studies*

According to the encouraging results obtained by KENAGA (1965), WOLFENBARGER et al. (1968), and SIDNEY (1968) on the effectiveness of tin compounds as inhibitors for egg production in insect, it was decided to carry out further investigations with Du-Ter in this direction.

Results obtained from the present work indicated quite clearly that Du-Ter acts as chemosterilant agent when applied to larval food. Egg production for moths obtained from treated larvae was reduced and viability of the eggs decreased. Such effect was negatively correlated with Du-Ter concentrations. These findings agree with those obtained by the previously mentioned workers. Also pupation percentage and pupal viability were adversely affected according to Du-Ter concentrations used in larval food.

Results obtained from applying the material directly to moths either by contact or orally in food, were agreable with those findings of several workers on moths (CHAMBERLAIN 1962, COLLER 1965, QUYE 1965, HENNEBERRY 1966, LADD 1966, TOPPOZADA 1966, SOTO 1967, and HENEIN 1968).

Generally, fecundity was affected in a negative relation with Du-Ter concentrations. Also sterility in eggs obtained was more observed in a positive relation with Du-Ter concentrations.

Comparing the relative potency of the material for both methods of applications on moths, it could be concluded that Du-Ter has higher relative potency when used orally in food than by contact. This was in harmony with results obtained with treated larvae.

3. *Histopathological studies*

The variation in mode and extent of action of the different insecticides on cotton leafworm *Spodoptera littoralis* BOISD. has been a subject of considerable discussion among toxicologists. Work with the mode of action of chemosterilant materials histopathologically on the cotton leafworm was relatively few. KISSAM et al. (1967) was the first one to study the effect of certain antifertility compounds on the house fly using histological technique.

Due to the very promising results obtained in the first and second part of this work, it was felt that histopathological studies were demanded to confirm the strong stomach action of Du-Ter.

Histopathological results indicated that Du-Ter was much more potent in destructing the tissues of the mid-gut, muscles, fat bodies than it was on Malpighian tubes. Such effect could furnish a concrete proof to the strong stomach action of Du-Ter.

The histopathological symptoms brought here were somewhat similar to those reported by SOLIMAN SOAD and SOLIMAN (1968).

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Ergebnisse über vergleichende Untersuchungen bei der Anwendung von Röntgenstrahlen und Chemosterilantien auf das Reproduktionssystem von *Rhagoletis cerasi* L.

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The effects of chemosterilants (Apholate, Tepa, Hempa) and gamma rays were compared under laboratory conditions on several developmental stages of cherry fruit fly, *Rhagoletis cerasi* L. The effective dosis and optimal exposure producing complete sterility with males and females were established, especially in case of Apholate. The irradiation with gamma rays produced similar effect in the reproductive system of flies as chemosterilants. According to behavioural studies the irradiation has to be applied very carefully, as often lesions in the optical sensory system were noticed, followed by abnormal behavioural pattern of the males, diminishing thus their possibilities in competition with wild populations. Studies carried out with coloured pans under conditions similar to field ones, using them in combinations with Apholate or Hempa, showed that methods of sterilisation could be used in larger scale against the cherry fruit fly. The success of the method depends on a suitable specific attractant, as the materials used (Buminal, Casein, Amyl acetate) were not satisfactory.

Im letzten Jahrzehnt ist besonders in den südlichen Kirschanbaugebieten der DDR eine beträchtliche Zunahme des Kirschfliegenbefalls zu verzeichnen. Für das Jahr 1969 beispielsweise wurden fast 50% des gesamten Aufkommens an Süßkirschen durch die Kirschenfliege »*Rhagoletis cerasi* L.« beeinträchtigt.

Die verwendeten Bekämpfungsmaßnahmen sind nur im beschränkten Maße von Erfolg gekrönt. Die zur Anwendung gelangenden Insektizide können aufgrund ihrer langen Karenzzeiten nicht voll wirksam eingesetzt werden. Dazu kommt, daß der Kirschenanbau in der DDR ein heterogenes Sortiment umfaßt und weiterhin durch den vielfach noch verbreiteten Unterbau an Beerenobst die Bekämpfung zusätzlich erschwert wird.

Im Zuge der sich in der DDR entwickelnden Maßnahmen zur integrierten Bekämpfung von Schädlingen im Obstbau wird auch nach neuen Wegen und Methoden zur Bekämpfung der Kirschfruchtfliege gesucht. Angeregt durch zahlreiche Beispiele des erfolgreichen Einsatzes der Autozidmethode zur biologischen Bekämpfung von Schadinsekten wurden im Deutschen Entomologischen Institut / Eberswalde Untersuchungen zur Sterilisierung der Kirschfruchtfliege durchgeführt.

Neben dem Studium der spezifischen ökologischen Verhältnisse unter den Anbaubedingungen unseres Landes wurden unter Laboratoriumsbedingungen

die Wirkung von Chemosterilantien (Apholate, Metepa, Tepa und Hempa) als auch die Einwirkung von Röntgenstrahlen untersucht.

Da sich die Kirschfruchtfliege für den allgemein üblichen Screening-Test, aufgrund ihrer geringen Generationszahl, nicht eignet und auch unter Laboratoriumbedingungen nur schwer züchten läßt, wurden die Untersuchungen anhand histomorphologischer Methoden durchgeführt.

Als Ergebnis umfangreicher Versuchsserien mit verschiedenen Dosierungen und Konzentrationen sowie Applikationsmethoden konnte für Apholate in 1-prozentig wäßriger Suspension unter Zusatz von 5% Bienenhonig eine Expositionszeit von 2 bis 3 Minuten als ausreichend für eine effektive Sterilitätserzeugung ermittelt werden. Metepa – als 0.75-prozentige Lösung – zeigte ebenfalls eine gute kontaktizide Wirkung und die Expositionszeit zur Erzeugung einer wirkungsvollen Sterilität beträgt 5 bis 6 Minuten.

Die genannten Chemosterilantien in der angewendeten Konzentration bewirken in den Hoden männlicher Fliegen eine Störung der Spermatogenese. Die histologischen Befunde ergaben eine deutliche Schädigung der Spermatocyten. Trotzdem werden noch bewegliche Spermien beobachtet, die im Ductus ejaculatorius (Samengang) als auch in der Bursa copulatrix des Weibchens nachgewiesen werden konnten.

Eine Störung des natürlichen Verhaltens der Männchen als auch der Weibchen konnte nicht beobachtet werden. Weibchen, die mit sterilen Männchen kopulierten, legten äußerlich völlig normal aussehende Eier, jedoch kamen diese nicht zur vollen Entwicklung.

1-prozentige bzw. 0.75-prozentige Konzentration von Apholate und Metepa rufen in den Ovarien der Weibchen ebenfalls deutlich sichtbare Veränderungen hervor. In den histologischen Schnittpräparaten sind deutlich erkennbar primäre Schädigungen des Follikelepithels wahrzunehmen. Im weiteren Verlauf kommt es zu einer Auflösung des Zellgewebes und zu einem Zerfall der Zellkerne. Daneben sind auch deutlich Schädigungen im Dottergewebe des embryonalen Eies sichtbar. Die so sterilisierten Weibchen sind also nicht in der Lage, intakte Eier abzulegen. Verhaltensstudien ließen keine Unterschiede zwischen sterilisierten und gesunden Weibchen erkennen.

Röntgenstrahlen erzielten in dem Reproduktionssystem männlicher und weiblicher Fliegen ähnliche Effekte wie chemosterilante Substanzen. Bestrahlt wurden Tönnchen etwa 40 Tage vor dem Schlupf. Dabei kamen Dosierungen von 500, 1000, 1500 usw. bis 5000 Röntgen pro Zeiteinheit zur Anwendung. Bereits nach 10 Tagen konnten die ersten Veränderungen vor allem im Imaginalgewebe der Prä-Puppe bzw. der späteren Puppe beobachtet werden.

Als Ergebnis der systematischen Untersuchungen über die Auswirkungen der verschiedenen Strahlendosierungen auf das Imaginal-Stadium anhand von Gewebeschnitten wurde festgestellt, daß bei männlichen als auch weiblichen Fliegen, in Abhängigkeit von der jeweiligen Strahlenmenge, eine Verkleinerung bzw. Verkümmerung der Gonaden eintritt. Etwa ab 4000 Röntgen ist eine völlige

Reduktion des Follikelepithels zu verzeichnen. Das Dottergewebe ist stark geschädigt und losgelöst vom Nährgewebe. Bei den männlichen Fliegen ist – ähnlich wie bei der Anwendung von Apholate – ebenfalls die Spermatogenese beeinträchtigt, die Spermatocyten sind teilweise völlig zerstört und die wenigen Spermien weisen anormale Deformationen auf. Trotzdem zeigten die Fliegen äußerlich in ihrem Verhalten anfänglich keine Abnormitäten. Nur konnte sehr selten eine Kopulation beobachtet werden. Genauere Untersuchungen brachten zu Tage, daß durch die Bestrahlung offensichtlich auch die sensorischen Organe Schädigungen erlitten hatten. Am auffälligsten war die abnorme depressive Ausbildung der Augen. Die Kristallzellen und Sinneszellen weichen von der normalen Morphologie ab und scheinen nicht voll ausgebildet. Ebenfalls ist eine Reduktion der Lamina ganglionaris sowie der Medulla externa und interna zu verzeichnen.

Weitere Studien ergaben, daß auch die tropotaktischen Organe einer Schädigung unterlagen. Leider ließen sich derartige, durch Röntgenstrahlen hervorgerufene Organschädigungen mit den zur Verfügung stehenden lichtoptischen Untersuchungsmethoden nicht exakt nachweisen.

In Fortführung der Untersuchungen wurden umfangreiche Experimente unternommen, die optimale Strahlendosis zur Erzeugung einer effektiven Sterilität zu ermitteln. Nach den vorliegenden Untersuchungsergebnissen, die durch Freilanduntersuchungen gestützt werden, ist eine Strahlungsdosis von 1500 bis 1600 Röntgen pro Zeiteinheit erforderlich. Diese Strahlendosis beeinflußt das natürliche Verhalten bei der Geschlechterfindung und Wirtsfindung offensichtlich nicht. Allerdings zeigten weitere Experimente mit einer größeren Zahl von Tieren unter annähernden Freilandbedingungen, daß hier nur eine partielle Sterilität, die zwischen 60 und 85% schwankt, erzeugt wird.

Ein Vergleich von Chemosterilantien und Röntgenstrahlen nach der beschriebenen Versuchsmethodik zeigt, daß Röntgenstrahlen anwendungs- und verfahrenstechnisch leichter und eleganter zu handhaben sind; andererseits lassen sich mit Chemosterilantien sichere Effekte erzielen, aber vor allem wird das natürliche Verhalten der Kirschfliegen anscheinend gar nicht und wenn, dann nur sehr unwesentlich beeinträchtigt. Aber nicht nur aus diesen genannten Erwägungen heraus ist der Chemosterilisations- oder der Bestrahlungs-Methode der Vorrang zu geben, sondern es spielen auch ökonomische Faktoren bei der breiten praktischen Anwendung der Autozidmethode zur Bekämpfung der Kirschfruchtfliege eine Rolle.

Zum Beispiel läßt sich unter den obstbaulichen Strukturverhältnissen in der DDR die Bestrahlungs-Methode zur großräumigen Bekämpfung der Kirschfruchtfliege nur schwer verwirklichen, da sie umfangreicher Laboratoriumszuchten bedarf, aber auch eines gut durchdachten, sehr aufwendigen Freilassungssystems. Auch dürfte beispielsweise die kontinuierliche Beschaffung der in die tausende gehenden Fliegentönnchen für die Massenzuchten aus ökonomischen Gründen problematisch werden. Deshalb haben sich unsere Untersuchungen auf die Methode der Sterilisation von Freilandpopulation, unter der Verwendung von spezifischen

Lockmitteln, konzentriert. Die Anwendung der Autozidmethode unter den Gesichtspunkten der Sterilisation der Freilandpopulationen mittels chemosterilanter Stoffe, kann unter Berücksichtigung geeigneter Verhaltensweisen der zu bekämpfenden Schädlingsart eine äußerst elegante und attraktive Methode sein.

Diese Überlegungen auf die Kirschfruchtfliege angewendet, zeigen, daß sehr reale Chancen bestehen, mittels dieser Praktiken eine erfolgreiche Dezimierung der natürlichen Populationen zu erzielen. Jedoch ist dazu unbedingt ein attraktiver Lockstoff erforderlich.

Anlockversuche mit der Kirschfruchtfliege sind schon seit etwa 60 Jahren bekannt. Neuere Arbeiten, besonders von HAISCH und FORSTER sowie BOLLER (1969), zeigen wohl einige interessante Gesichtspunkte, jedoch sind noch keine wirkungsvolle Techniken erarbeitet worden.

Wir haben auf der Grundlage der von den Autoren genannten Untersuchungsergebnisse ähnliche Versuche durchgeführt. Dazu wurden als Ergebnis langjähriger Experimente gelborange farbige Ködertafeln in den Abmessungen 20×20 cm, die mit einer Apholat-Honig-Suspension versehen waren, zur Anköderung von Kirschenfliegen benutzt.

Im Prinzip konnte mittels dieses Verfahrens eine wirkungsvolle Sterilisation der Kirschenfliegen erzielt werden. Unbefriedigend sind allerdings zur Zeit die geringe Anzahl von Fliegen, die diese Ködertafeln anfliegen. Vorsichtige Schätzungen in mehreren Freilandversuchen – die zum Teil mit eingezwingerten Kirschenfliegen durchgeführt wurden – ergaben Anflugerfolge zwischen 25 und 33%. Versuche mit Amylacetat, Ammoniumstearat, Ammoniumkarbonat sowie Anisöl und Angelikaöl brachten keine wesentlichen Verbesserungen. Es bleibt weiteren Untersuchungen vorbehalten, attraktivere Stoffe zu ermitteln.

Zusammenfassung

Unter Laboratoriumsbedingungen wurde vergleichend die Wirkung von Chemosterilantien (Apholate, Tepa, Hempa) und Röntgenstrahlen auf verschiedene Entwicklungsstadien der Kirschfruchtfliege untersucht. Als Ergebnis konnte die effektive Wirkung besonders von Apholate sowie die Sterilität erzeugende optimale Expositionszeit für Männchen und Weibchen ermittelt werden. Die Anwendung von Röntgenstrahlen erzielte in dem Reproduktionssystem der Fliegen ähnliche Effekte wie chemosterilante Substanzen. Verhaltensphysiologische Untersuchungen haben gezeigt, daß besonders Röntgenstrahlen sehr differenziert angewendet werden müssen, da oftmals eine Schädigung der optisch sensorischen Organe eintritt, die das natürliche Verhalten der Männchen beeinflussen und somit die Konkurrenzfähigkeit gegenüber Freilandpopulationen herabmindern.

Versuche unter freilandähnlichen Bedingungen mit farbigen Ködertafeln, in Kombination mit Apholate oder Hempa weisen auf die Möglichkeit hin, die

Sterilisierungsmethode auch im größeren Umfang zur Bekämpfung der Kirschfruchtfliege einzusetzen. Diese Methode ist abhängig von einem spezifisch wirkenden effektiven Attraktivstoff, die verwendeten Köderstoffe Buminal, Casein, Amylacetat befriedigen zur Zeit nicht.

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Possibilities of the Genetic Control Against the European Corn Borer (*Ostrinia nubilalis* Hb.) in Hungary

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The European corn borer (*Ostrinia nubilalis* Hb.), a native and common pest in Hungary, has several hosts both among the cultivated and wild plants. Its presence as a pest is most perceptible on maize in the S—SE part of the country. An extended, two-peaked flight period has been shown mainly in S-Hungary by light traps, however, without an exact proof of a second brood. Therefore, it would be sufficient to carry out the possible biological (incl. genetical) control at the time of the first flight (peak) only. There are no exact data on the population density in nature. However, basing mainly on the number of overwintering larvae in corn stalks the population density may be estimated as 1000 adults per ha in the average. Rearing of more than 40 consecutive generations without diapause on a simplified artificial diet has been achieved, after this, however, the viability of the eggs decreased. As far as the sterilization is concerned, there seems to be no special difficulty, as shown also by earlier authors. Mortality of 80—97 per cent has been found in eggs laid by females, mated with males irradiated (30 kr gamma) in the pupal stage. The irradiation of young adults seems to be more suitable, because of the high mortality of irradiated pupae. The applicability of substerile doses related to the corn borer should be tried in the near future. There are practically no experiences yet with the field release of sterilized corn borer adults. Because of the large corn areas, it could be hardly carried out without aerial application, however, owing to the relatively strong dispersal of the adults, fewer release points may be needed compared e.g. to the codling moth. The release period should cover about ten weeks. Calculating with a ratio 10 : 1 of the sterilized moths to the native ones and with the above mentioned abundance, it means a need for 10.000 sterilized moths per hectare. The genetic control method may be considered as a part of the integrated control. In the case of using a dense network of release points, the moth release could be combined also with release of *Trichogramma*.

In accordance with problems in Hungary in the pest control and with the recommendation of the different IAEA panels (e.g. CUTCOMP, 1967), we started research in the field of the sterile-male technique with the cockchafer (JERMY and NAGY, 1967), codling moth (JERMY and NAGY, 1970) and bean weevil (JERMY, 1970). These insects will remain the main projects, in this respect, in the next future too.

Among the further possibilities there is also the corn borer (*Ostrinia nubilalis* Hb.), which seems to present considerable difficulties mainly because of

the great, adjacent infested areas and possible large abundance from time to time. However, the huge number of investigations and literature data in Hungary and foreign countries make it possible to conduct a general survey, regarding the possibilities of genetic control of the corn borer.

Distribution and Host Plants

The corn borer is a native, overall common insect pest in the Carpathian Basin. One of its main host plants originally might have been the wild hop *(Humulus lupulus)*, which is very common in the alder-groves (plant community *Alnetum* and their fragments). The wild-hop used to be infested with corn borer very often, however, for the last century the corn *(Zea mays)* should be regarded as principal host plant. Beside this there are several wild and cultivated plants, which may serve as primary or secondary hosts; therefore, the Great Hungarian Plain represents for the corn borer a large continuous breeding-area. This large, nearly unbroken breeding-area represents the biggest difficulty also for the genetic control.

Damage

The Hungarian corn growing area belongs to the secondary damaged zone and the stalk infestation by the corn borer larvae seldom exceeds 50 per cent of the stalks. The damage is the most perceptible in the southern and south-eastern parts of the country, which is the main corn growing area. Some restricted damage may occur on cultivated hop (BENEDEK et al. 1966) and on hemp (NAGY and CSEHI, 1955) as well. The total loss caused by the corn borer in Hungary may be estimated between 2.5 and 20 million $ per year.

Classical Control Methods

Among the cultivated host plants the hemp, the hop, but in the first line the corn might need control measures. The corn area in Hungary (1.268,000 ha in 1969) represents one quarter of the total crop area. Several insecticides proved to give good control against the young corn borer larvae (NAGY, 1962; NAGY and PÁSZTOR, 1962), but at present the costs of control might be near to the economic level. Also the hazards should be taken into consideration, which the enormous big treated area would represent in the case of using insecticides.

In the practice, at present, the means of control are limited to agricultural, mechanical methods, namely to stalk-destroying, which should be finished until the beginning of the flight (until the middle of May). However the fulfilment of the stalk-destroying is occasionally insufficient. Some hybrid-corn varieties grown in Hungary show different degree of resistance or tolerance against the corn borer (DOLINKA, 1962), however it is not high enough yet to avoid damages.

Population Density

Several examinations were carried out concerning the larval abundance, but not the adult abundance. This latter would be more important in view of the sterile male release. The light trap data give only relative clues for the moth abundance in the field and even the number overwintering in the corn stalks gives hardly utilizable data because of different mortality rates resulting from stalk-destroying, parasites, etc.

So in 1967 in Felsőnyomás (SE-Hungary) 0.35–0.72 borer was found per stalk at a 35–72 per cent stalk infestation, however, in the other years only the half of this value. But the presence of 0.25 borer/stalk means still 10,000 borer/ha (based upon 40,000 plant/ha). Now, taking these 10,000 fullgrown borers per hectare and calculating roughly with a 60 per cent mortality, caused by the agricultural techniques (stalk-destroying, ploughing etc.) mainly in autumn, and further with a 15 per cent mortality, caused by biotic factors (entomophags, diseases), and with 15 per cent mortality, caused by different abiotic factors mainly during the diapause (overwintering), we may count at the end with about 1,000 moths per hectare. This number may be considered as a general average for the corn growing area during the first flight period.

Flight, Number of Generations

Generally speaking, at the sterile male release work, the presence of the native adults in the field determine the period and amount of the release. Detailed investigations have shown that in Hungary the corn borer adults may occur from middle of May to the end of September, however with two, relatively well-separated flight peaks mainly in the southern half of the country; the first in June–July (spring-flight), the second in August (summer-flight; NAGY, 1960, MÉSZÁROS, 1969).

It is a strange phenomenon that until the present time there is no exact proof for the existence of a second brood in Hungary. The sometimes numerous adults of the second flight disappear without starting a new (second) generation (NAGY, 1961; 1964). Thus, a possible sterile insect release should be timed against the first flight only. This relatively short period required for releasing no more as ten weeks, may be important and to some extent facilitates a possible release programme against the corn borer. At this point we can refer to the releasing period of five months required against the codling moth (PROVERBS et al. 1969). However this ease, the shorter releasing period at the corn borer, may have to be taken into consideration in the one-generation area only.

There seems to be no difficulty in the signalization of the adult flight of the *Ostrinia nubilalis* (NAGY, 1960, 1961, 1964; MÉSZÁROS, 1969) because of the well organized light trap system in Hungary.

Mass Rearing

There are several types of diets for rearing corn borer larvae, but most of them seem to be too expensive and complicated for an economic mass rearing.

By means of a simplified semiartificial diet we have succeeded in rearing over 40 consecutive generations without diapause (NAGY, 1970); however, at some later generation there appeared a decrease in the viability of the eggs, supposedly the same phenomenon which HUGGANS and GUTHRIE (1970) have found in their rearings. This can be avoided surely by refreshing occasionally the laboratory stock. Further research is needed also to improve economy and automation of mass rearing techniques.

Compared to the codling moth we may count with a higher demand for space in the mass-rearing on artificial diets. The larvae of the corn borer are about twice as large as the codling moth and they have also little inclination for cannibalism. We succeeded to rear specimens with a minimum space of 8 – 10 ccm per individual.

Sterilization

Earlier studies have shown the possibility of the sterilization by irradiation. WALKER and BRINDLEY (1963) got high level of unfertile eggs from normal females after mating with sterilized males exposed to 32,000 krad X-rays; ANWAR (1968) got nearly the same level using 40 krad γ-rays (Co-60) on older pupae.

In preliminary experiments – treating pupae with 30 krad γ-radiation – we have got 80.3 and 97.4 per cent unfertile eggs after caging normal females with sterilized males, in equal numbers, however the data of the replications varied between 76 and 100 per cent. WALKER and BRINDLEY and ANWAR (1963) have found different rates of pupal mortality caused by irradiation; the same proved to be 53, and 44 per cent, resp. in our two experiments using 30 krad gamma radiation on pupae of mixed age.

Therefore the sterilization by irradiation should be aimed against young adults, however, the handling of the relatively large moths would represent further difficulties.

Based on the papers of WALKER and BRINDLEY and ANWAR mentioned above, the competitiveness of the sterilized males seems to be sufficient for the sterile male release procedure.

The possibility for using chemosterilants to sterilize European corn borer was not yet proved. However it seems to be a relatively simple way for this purpose, namely using chemosterilants solved in the drink-water of adults, as both the males and females of the corn borer drink water.

Release

There are practically no experiences yet on the field release of sterilized corn borer adults. Based on the calculated 1000 moth per ha for the first flight, i.e., in the average 500 males per ha, 5000 sterilized males, or (in the case of sexually mixed populations) 10,000 moths would be needed per hectare, to achieve a ratio of 10 : 1. Calculating with a ratio of 20 : 1, or 30 : 1, the needed amount of sterilized moths is nearly the same which PROVERBS et al. (1969) have used against the first brood of the codling moth in 1968 in Canada. However, in the case of the corn borer, this same amount of sterilized moths should be released during a much shorter period and on a much larger area, than it was necessary in the case of codling moth.

According to the specific circumstances of the population ecology of the corn borer in Hungary, the period of release should cover no more than 10 weeks in May – June – July, namely the period of the first (spring) flight. This relatively short period required for release may consider an important ease regarding the release work, but a difficulty for the rearing.

Because of the large corn areas in Hungary, the release could be hardly carried out without aerial application, however – owing to the relatively stronger dispersal of the adults – scattered release points may be needed compared e.g. to the codling moth.

Related Possibilities, Integrated Control

The genetic control as a possible future method may be considered as a part of the integrated control in the reduction of corn borer populations. Efforts must be made to improve the stalk handling and for using more and more resistant corn hybrids (DOLINKA, 1968).

In the case of using a more dense network of release points, the release of sterilized moths could be combined with release of *Trichogramma* the latter for controlling the remained viable corn borer eggs by egg-parazitization. We may mention further the theoretically possible application of the laboratory-reared multivoltine-strain against the univoltine field-strain of the corn borer – a genetic method in a narrow sense – to disturb the diapause ratio.

There are some successful field experiments in using the egg parasite *Trichogramma* to control corn borer eggs (e.g. ZIMIN and KIVIT, 1936; GUSZEVA, 1963), but an application of multivoltine strains against a univoltine strain was not proved yet.

The possibility for using the substerility principle against the corn borer, is also a question not yet settled.

As a final conclusion, owing to the tremendous amount of information on the biology and ecology of the corn borer and meeting most of the main

requirements of the sterile male technique, joined to the summarized results of LINDQUIST (1963) we may consider the European corn borer as a possible and required object for further, detailed research of the genetic control methods.

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Effect of Fungicides and Insecticides on the Biological Activity of *Bacillus thuringiensis* Berl. Preparations

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A laboratory method has been worked out for testing pesticides for compatibility with *B. thuringiensis* Berl. preparations. It was stated that most insecticides are compatible with *B. thuringiensis* if they are not used in higher concentrations than recommended for practical use and the two components are mixed shortly before spraying. Most fungicides studied (coppersulfate, Dexon, Karathane, Difolathan) decreased the effectivity or spore viability. In large scale field experiments against the fall webworm, *Hyphantria cunea* Drury, insecticides (Ditrifon, Gusathion, Imidan, Lebaycid, malathion, Nogos, phosphamidon, Ultracid) showed not only good compatibility with *B. thuringiensis* but also increased the effectivity of the combinations, although the insecticides were used in one-tenth of the concentration recommended in the practice. So it was possible to lower also the concentration of *B. thuringiensis*, reducing thus the costs of treatment.

As a result of undesirable consequences of chemical pest control an ever increasing attention has been paid in the last decade to selective methods and biological control (Herfs, 1968a), among which the use of *Bacillus thuringiensis* preparations played a prominent role. Considering the wast amount of data, it may be sufficient to refer to some comprehensive studies published in this field (Herfs, 1967, 1968b, Cameron, 1968, Weiser, 1970) in which the authors have pointed out not only the benefits resulting from the selective use of the bacterium against a long list of lepidopterous pests, but also reviewed some disadvantages as well, e.g. the unfavourable physical properties of some preparations (rapid setting of spore suspensions, clogging of spray nozzles) and the high costs of preparations. The standardization problems of manufacturing and the difficulties in practical use may be regarded as reasons why *B. thuringiensis* preparations have not become more common tools of plant protection in many countries, as in Hungary too, in spite of the early pioneer work carried out here with this pathogen (Husz, 1931, Klement, 1953).

The needs of controlling non-susceptible pests attacking simultaneously with susceptible *Lepidoptera* and the efforts to reduce treatment costs, brought with themselves many experiments to study the compatibility of *B. thuringiensis* preparations with several pesticides and adjuvants. Hudon (1962) found the combination of *B. thuringiensis* and Sevin superior to Sevin alone, applied in

a higher concentration; MARTOURET (1959) tested successfully *B. thuringiensis* combined with dithiocarbamate and with copperoxychloride. MCEVEN et al. (1960) found the *B. thuringiensis* combinations with Sevin, Demeton, TEPP, Trithion, Maneb, Captan, Diodine, Ferbam, Glyodine and parathion effective, whereas Choranyl decreased the activity of the bacterium. In the experiments of FEDORINCHIK (1964) sublethal doses of DDT, DNOC, Kelthane, Tyophos, Phygon, Cyram and anabasin sulphate gave good results combined with *B. cereus*, related to *B. thuringiensis;* BHC and Bordeaux mixture however reduced the activity of the pathogen. TELENGA (1964) used succesfully *B. cereus* combined with low-dosage methylparathion and DDT against various lepidopterous pests. In his comprehensive work based partly on the sources mentioned above, HERFS (1965) stated that *B. thuringiensis* may be combined without loss of biological activity with most insecticides, fungicides and adjuvants, with the exception of Bordeaux mixture, Glyodin, Dodine, Choranyl, BHC, Diasinon, malathion, TEPP, various carbamates, polyvinylalcohol, Lovo 192, Bentonite, sodium sulphate and sodium hypochlorite (the latter five being adhesives or carriers). In the publication of the International Minerals and Chemicals Corporation (ANONYMUS, 1969) 58 preparations are listed (insecticides, fungicides, adjuvants) which are compatible with Thuricide products in case of regular use (prompt use after mixing); only Glyodine is mentioned as incompatible.

In our own experiments we aimed to study the effects of several pesticides on *B. thuringiensis*, under laboratory and field conditions. In the 5 year's research the fall webworm, *Hyphantria cunea* has been used as test animal, partly due to its importance as a quarantine pest, partly owing to the fact that the use of *B. thuringiensis* seems to be perspectivic against this species. *H. cunea* appears in high individual density mostly in the autumn in Hungary (second generation) and the use of pesticides in orchards becomes hazardous at that time of the year.

Materials and Methods

a) The first experiments were carried out in 1966 in the Department of Invertebrate Pathology, University of California (Berkeley). The inhibitory effect of some commercial pesticides has been studied under laboratory conditions, by exposing the *B. thuringiensis* spores to various dilutions, then evaluating the effect by counting the number of colonies developed on the surface of nutrient agar plates. In the first series we diluted the various pesticides (listed in Table 1) to 1, 0.5 and 0.1 % "spray liquids" and then added to each test tube 1 ml spore suspension (made by a 10^6 dilution from the "Thuricide 90 TS" commercial preparation, which contains 30 billion viable spores per 100 gram). After thoroughly mixing by using sterile glass rods (to prevent the setting of spores to the bottom), 0.1 ml of each suspension was spread onto nutriens agar plates (3 Petri dishes for each variation) and kept at 28 centigrades for 48 hours. The evaluation was

carried out after 48 hour's incubation, by counting the numbers of colonies on 3 sq.centimeters selected at random in each Petri dish (9 measurements in each variation). This experimental series failed however, as owing to the high amount of pesticides present on the agar surface, no colonies developed (except in the distilled water control).

So the amount of pesticides spread onto the plates had to be reduced; in the second series we added a much higher amount of spore suspension to the 1, 0.5 and 0.1 % "spray liquids", then took from them 1 ml homogenized samples after 4 and 48 hours. These samples were then diluted to a high degree with sterile distilled water, so the end dilutions became 0.0001 – 0.00001 % for the pesticide, according to the initial concentration. By spreading 0.1 ml of this diluted suspension onto nutrient agar plates, each sq. centimeter showed 0 – 300 colonies after 48 hours of incubation (28 Centigrades), according to the grade of inhibition. The data given in the paper are per cent values of decrease in colony numbers compared to the dist. water controls, counted from 9 observations in each variation. The pH values of the "spray liquids" were also measured 4 and 48 hours after the beginning of the experiment.

b) These experiments were then continued in 1967 in Hungary with the same pesticides (plus some additional ones as listed in Table II.), by using L_4 *Hyphantria cunea* larvae as test animals. The Thuricide 90 TS spore suspension was added to the 1 % "spray liquids", then after 24 hours we made a thousandfold dilution; so the resulting "spray liquid" became 0.2 % for *B. thuringiensis* preparation and 0.001 % for the given pesticide. In separate parallel experiments the pesticides were tested alone in the same concentration (0.001 %) for effectivity. The larvae were kept in groups of 50 on separate apple twigs, with 3 repetitions in each variation. The spraying was carried out with a hand sprayer, by using the same amount of spray; the larvae were placed to the foliage after drying up. The larval mortality was evaluated after 96 and 120 hours.

c) The experiments mentioned in sections *a)* and *b)* gave enough informations for the field experiments, carried out through 4 consecutive years (1967 – 1970). As the methods used were the same during this period, they can be summarized.

In the four years mentioned, the field experiments were carried out by the aid of a District Plant Protection Station (Fácánkert) in the area of Szedres-Medina (Central Hungary) on roadside maulberry rows heavily infested by *Hyphantria cunea*. The sprayings were done each year at the time of the second generation, in September, on larval populations consisting of $L_4 - L_6$ instar larvae. The larval instars were established in each experiment by measuring the width of head capsules. The sprayings were done every year by using a large output mist-blower spraying machine (Type Rapidtox Super RS-N) and by spraying 6.5 liter spray on each full grown (10-year-old) tree. The *Hyphantria* "nests" (larvae of the same egg batch live together until the end of their L_5 instar) were isolated into cheesecloth isolators on the sprayed trees, then cut down after 24 hours together

Table 1

Reduction in number of colonies of *B. thuringiensis*—as compared to the dist. water control— after exposure to different pesticides (Berkeley, 1966)

Pesticide	Concentration %	% Reduction after exposure of		pH of "spray liquid" after 4—48 hours
		4 hours	48 hours	
DDT	1	48±7.6	56±9.2	
	0.5	25±4.9	52±6.4	4.97—5.06
	0.1	5±2.3	42±6.2	
Sevin	1	47±6.7	52±5.4	
	0.5	16±2.1	43±6.9	4.95—5.07
	0.1	6±0.3	43±3.7	
Parathion	1	37±0.9	80±7.3	
	0.5	10±1.2	63±7.1	4.48—4.54
	0.1	1±0.1	58±2.9	
Malathion	1	33±4.6	57±2.6	
	0.5	13±4.3	39±4.1	2.85—3.10
	0.1	5±2.3	25±1.1	
Phosphamidon	1	23±2.2	52±2.3	
	0.5	13±0.1	50±2.1	2.67—2.70
	0.1	2±0.1	39±0.5	
Karathane	1	33±6.9	55±1.1	
	0.5	22±7.1	40±0.3	6.23—6.82
	0.1	4±3.2	30±0.3	
Dibrom	1	50±1.2	90±6.6	
	0.5	22+3.4	86+6.5	2.42—2.43
	0.1	5±1.1	70±4.9	
Captan	1	6±1.2	22±5.3	
	0.5	2±1.2	12±6.5	6.73—7.43
	0.1	1±0.1	5±0.2	
Maneb	1	8±0.7	37±0.8	
	0.5	5±0.1	30±1.2	8.23—8.80
	0.1	1±0.1	5±0.2	
Panogen	1	35±4.2	53±0.9	
	0.5	21±2.1	51±0.8	8.40—8.50
	0.1	5±0.1	36±1.1	
Dexon	1	6±0.6	8±1.2	
	0.5	2±0.1	6±0.1	6.30—6.42
	0.1	2±0.1	1±0.1	
Coppersulphate	1	53±6.2	68±1.1	
	0.5	22±2.2	46±0.8	4.30—4.32
	0.1	12±0.8	21±0.5	
Difolathan	1	100—	100—	
	0.5	100—	100—	7.19—7.90
	0.1	99.9—	100—	
Control (dist. water)	—	—	—	6.13—6.82

with the foliage the larvae were feeding on. From each variation 3 isolators were taken and their larvae kept separately until the end of the experiment. From the 72nd hour on, the larvae were fed with new leaves taken from the sprayed trees of the particular variation. The evaluation of mortalities was carried out 24 hours after the spraying and then subsequently in the 48th 96th 120th and 144th hour. In some years the evaluation in the 96th or 120th hour had to be omitted from technical reasons. In the tables and figures the standard mean deviations resulting from the 3 repetitions in each variation are given at the closing date of the experiment. The degree of infestation and the high number of trees (60–100 trees) treated in each variation made possible that the evaluation be carried out on 1500–2000 larvae per variation.

In the large scale experiments the spraying was effectuated shortly after the mixing of pesticide and *B. thuringiensis* preparation, with the exception of the experiment in 1968, where the *B. thuringiensis* suspension and pesticides were sprayed onto the same trees separately. In the field experiments we used the spore suspension Thuricide 90 TS and in 1970 also the preparation Biotrol WP. The pesticides used in the different experiments are listed at the end of the paper; the concentrations given refer to active material.

Results

By using the methods described in section *a)*, according to the treatment, small (0.1–0.3 mm diameter) white colonies developed on the agar plates; the percentages of their relative number compared to the untreated control (suspension + dist.water) are given in Table 1.

As it may be seen from Table 1 the inhibitory effect (reduction in number of colonies) was proportional to the concentrations and to the lenght of exposure. A longer exposition to a given pesticide may not happen in the practice, but under conditions similar to the ones of plant protection practice, an inhibition over 20% was noticed in the case of DDT, Karathane, Dibrom, Panogen, copper-sulphate and Difolathan. Especially the last mentioned exerted a high inhibitory effect, which could be showed also in repeated experiments even in case of 0.1%. There was no significant correlation noticed between the pH values of the "spray liquids" and grades of inhibition.

In the experiment *b)* the same pesticides (plus Nogos, Ultracid, DL-40, Lebaycid, Chlorbenzilate) were tried for their influence on *B. thuringiensis* activity, but measured on *Hyphantria* larvae; in Table 2 only the mortalities caused by the *B. thuringiensis* + pesticide combinations were given in the 96th and 120th hour, as the pesticides used alone in 0.001% concentration did not cause mortality.

The organophosphate insecticides did not seem to decrease *B. thuringiensis* activity, even under the "forced" conditions of the experiment (higher concentra-

Table 2

Effect of pesticides on the biological activity of *B. thuringiensis* (Laboratory experiments with L_1 *Hyphantria* larvae, Budapest, 1967)

Thuricide + pesticide mixture	Mortality after treatment, %	
	96 hours	120 hours
Thuricide 0.2% alone	97.2±0.2	100.0
Th. + Phosphamidon	100.0	—
Th. + Malathion	100.0	—
Th. + Nogos	100.0	—
Th. + Ultracid	97.8±0.1	100.0
Th. + DL-40	97.4±1.1	100.0
Th. + Parathion 20	93.4±2.2	97.9±0.1
Th. + Lebaycid	88.0±0.9	100.0
Th. + Captan	86.8±1.2	96.5±0.2
Th. + Maneb	93.4±0.9	92.7±1.2
Th. + Sevin	73.1±0.7	87.7±1.3
Th. + Coppersulphate	65.7±3.4	76.2±2.6
Th. + Dexon	52.4±8.1	79.7±1.3
Th. + Karathane	38.5±6.1	66.7±2.8
Th. + Difolathan	38.3±4.4	75.6±1.6
Th. + Chlorbenzilate	22.6±1.1	99.7±0.1
Control (untreated)	—	—

tion of pesticide than recommended, long exposure); most fungicides however retarded or reduced the effectivity of *B. thuringiensis*, as the mortalities showed in the 96th hour.

In the first year of the large scale experiments (1967), Thuricide was sprayed in 0.1 and 0.2% alone and combined in 0.1% with Nogos and Ultracid insecticides, the latter two in 0.02%. The results are shown in Table 3 and Fig. 1.

Table 3

Results of field experiment (*Hyphantria cunea*, Szedres-Medina, 1967)

Treatment %	% Mortality after						
	24	48	72	96	148	172	hours
Thuricide 0.1	5.9	45.6	47.2	54.3	55.9	60.6	±4.2
Thuricide 0.2	6.5	42.1	55.1	67.1	70.3	79.8	±5.3
Th. + Ultracid 0.02	62.1	92.1	93.5	94.5	98.3	100.0	—
Th. + Nogos 0.02	62.8	94.2	95.1	100.0	—	—	
Ultracid 0.02	5.2	20.3	31.9	33.0	47.2	38.9	±6.2
Nogos 0.02	5.3	22.5	37.2	46.5	58.3	63.2	±8.4

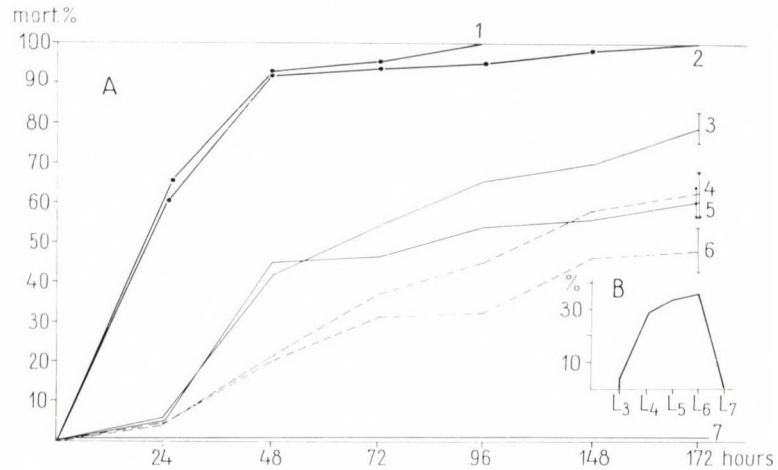

Fig. 1. Results of field experiment (Szedres-Medina, 1967). A = % mortality of *Hyphantria* larvae in 24—172 hours following the treatment; 1 = Thuricide 0.1 + Nogos 0.02%; 2 = Thuricide 0.1 + Ultracid 0.02%; 3 = Thuricide 0.2%; 4 = Nogos 0.02%; 5 = Thuricide 0.01%; 6 = Ultracid 0.02%; 7 = Control. B = Composition of larval population from point of view of developmental instars

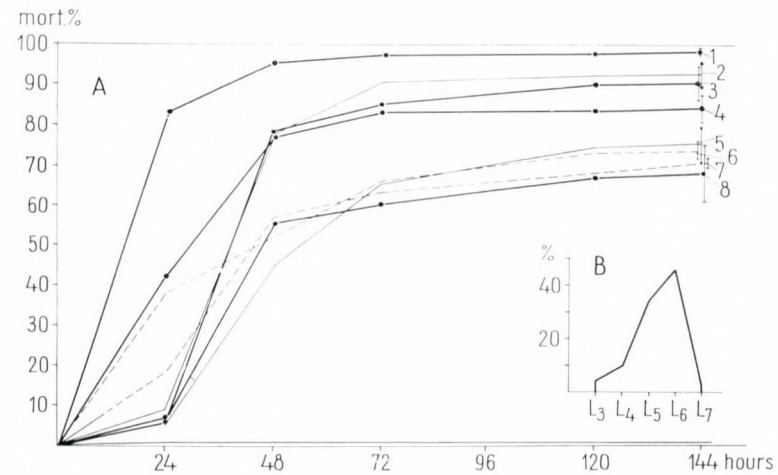

Fig. 2. Results of field experiment (Szedres-Medina, 1968). A = % mortality of *Hyphantria* larvae in 24—144 hours following the treatment: 1 = Thuricide 0.2 + Gusathion 0.01%; 2 = Thuricide 0.3%; 3 = Thuricide 0.2 and Gusathion 0.01% sprayed separately; 4 = Thuricide 0.2 + Sevin 0.07%; 5 = Thuricide 0.2%; 6 = Gusathion 0.01%; 7 = Sevin 0.07%; 8 = Thuricide 0.2 and Sevin 0.07% sprayed separately; 9 = Control. B = Composition of larval population from point of view of developmental instars

It became obvious that under field conditions the concentration of Thuricide cannot be lowered under 0.2% without a loss of effectivity; the insecticide combinations however increased the effect of bacteria in spite of their low concentrations, which were not effective enough if the insecticides were used alone.

In the second year (1968) we have studied the synergistic effect not by mixing the two components but by spraying them separately. In this year Gusathion (0.01%) and Sevin (0.07%) were used in combination with Thuricide (0.2 and 0.3%). The results were shown in Table 4 and Fig 2.

Table 4

Results of field experiment (*Hyphantria cunea*, Szedres-Medina, 1968)

Treatment %	% Mortality after				
	24	48	72	120	148 hours
Thuricide 0.2	5.7	46.8	66.6	75.9	77.6±6.2
Thuricide 0.3	9.2	77.7	90.5	92.7	93.2±4.1
Gusathion 0.01	37.9	52.6	67.1	74.3	74.9±5.1
Thuricide 0.2 + Gusathion 0.01 mixture	83.5	95.8	97.8	98.2	98.7±1.5
Thuricide 0.2 + Gusathion 0.01 separate	6.3	78.0	85.7	90.0	91.5±7.6
Sevin 0.07	18.7	57.0	63.4	67.9	68.3±1.3
Thuricide 0.2 + Sevin 0.07 mixture	42.9	78.4	83.4	83.9	84.2±7.6
Thuricide 0.2 + Sevin 0.07 separate	5.9	55.5	60.3	67.4	68.9±9.1
Control	—	—	—	—	—

Against the L_6 instar larvae Thuricide gave in 0.3% good results whereas the less satisfactory effect of the lower (0.2%) concentration could be substantially increased by pesticide combinations. There was no significant difference between the combinations and the separate use of bacteria and pesticides.

In the third year (1969) Thuricide was used again in 0.3 and 0.2% and combined with Ditrifon 0.02; the latter concentration was one-tenth of the one

Table 5

Results of field experiment (*Hyphantria cunea*, Szedres-Medina 1969)

Treatment %	% Mortality after				
	24	48	72	120	144 hours
Thuricide 0.2	39.4	49.6	64.6	73.1	76.8±4.6
Thuricide 0.3	40.0	69.9	78.7	83.8	85.4±2.3
Ditrifon 0.2	93.0	99.5	99.9	100.-	—
Ditrofon 0.1	82.6	94.5	96.9	98.4	98.7±0.6
Ditrifon 0.02	53.6	55.2	59.5	61.3	61.6±8.2
Thuricide 0.2 + Ditrifon 0.02 mixture	96.1	97.9	99.3	99.6	99.7±0.1
Control	—	—	—	—	—

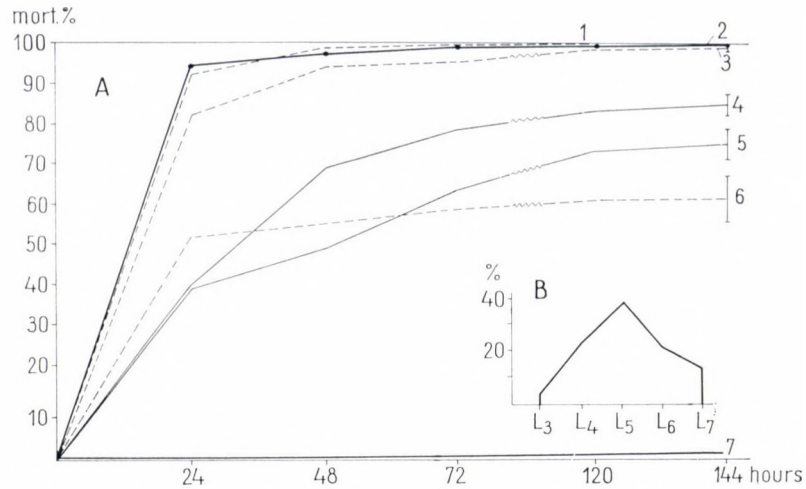

Fig. 3. Results of field experiment (Szedres-Medina, 1969). A = % mortality of *Hyphantria* larvae in 24—144 hours following the treatment; 1 = Ditrifon 0.2%; 2 = Thuricide 0.2 + Ditrifon 0.02%; 3 = Ditrifon 0.1%; 4 = Thuricide 0.3%; 5 = Thuricide 0.2%; 6 = Ditrifon 0.02%; 7 = Control. B = Composition of larval population from point of view of developmental instars

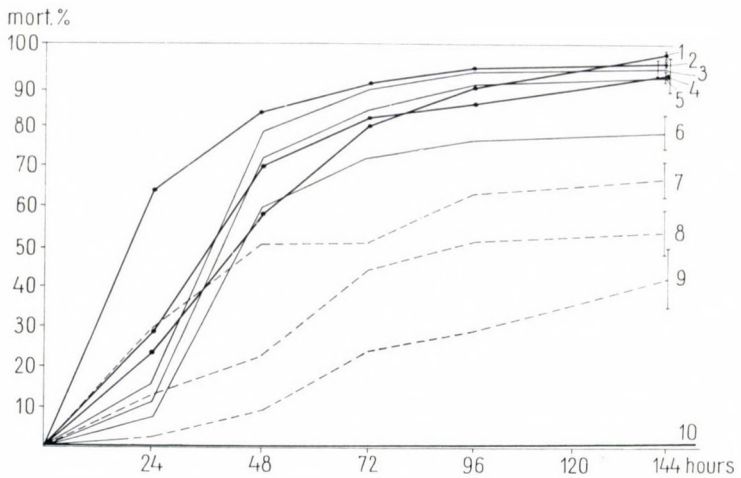

Fig. 4. Results of field experiment (Szedres-Medina, 1970). A = % mortality of *Hyphantria* larvae in 24—144 hours following the treatment; 1 = Biotrol 0.2 + Diasinon 0.02%; 2 = Biotrol 0.2 + Imidan 0.02%; 3 = Biotrol 0.3%; 4 = Biotrol 0.2 + Pirimor 0.02%; 5 = Thuricide 0.3%; 6 = Biotrol 0.2%; 7 = Diasinon 0.02%; 8 = Pirimor 0.02%; 9 = Imidan 0.02%; 10 = Control

Table 6

Results of field experiment (*Hyphantria cunea*, Szedres-Medina, 1969)

Treatment %	% Mortality after				
	24	48	72	96	144 hours
Thuricide 0.3	11.6	72.3	84.9	92.4	93.4±5.2
Biotrol 0.3	16.1	79.1	91.6	94.8	96.9±2.3
Biotrol 0.2	7.8	60.4	72.9	76 2	78.7±5.3
Biotrol 0.2 + Diasinon 0.02 mixture	24.9	58.5	80.6	91.2	98.6±1.1
Biotrol 0.2 + Imidan 0.02 mixture	66.1	84.9	92.2	95.8	97.9±0.7
Biotrol 0.2 + Pirimor 0.02 mixture	29.2	70.6	82.2	87.5	93.9±1.2
Diasinon 0.02	29.1	52.0	52.2	63.9	67.3±4.9
Imidan 0.02	3.5	9.5	24.4	29.3	43.7±7.6
Pirimor 0.02	12.4	22.6	44.9	51.8	53.6±6.5
Control	—	—	—	—	—

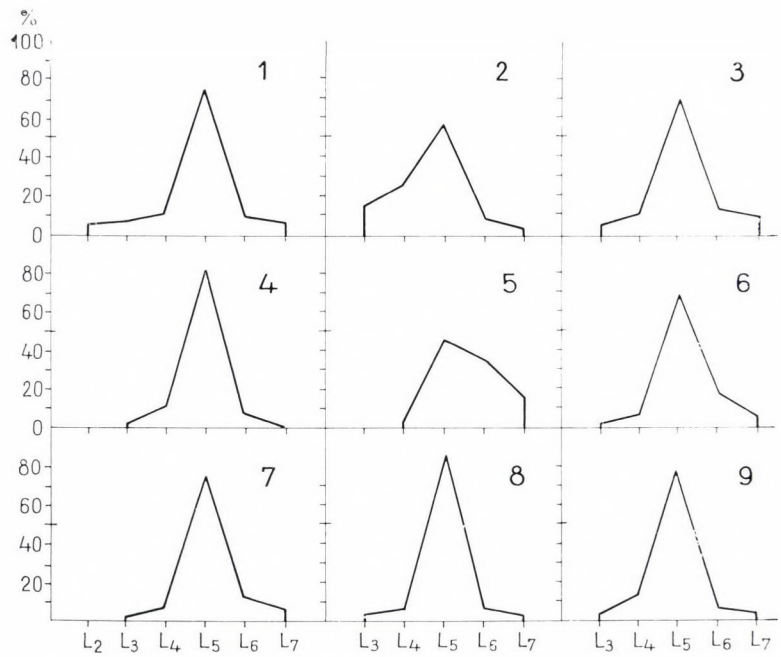

Fig. 5. Composition of the larval population from point of view of developmental instars, treated in the 1970 field experiment. 1 = Biotrol + Pirimor; 2 = Thuricide; 3 = Pirimor; 4 = Diasinon; 5 = Imidan; 6 = Biotrol + Diasinon; 7 = Biotrol + Imidan; 8 = Biotrol 0.3%; 9 = Biotrol 0.2%

used in practice. The results (also the ones achieved with Ditrifon alone) are shown in Table 5 and Fig. 3.

Thuricide showed a lower effectivity in the 0.3% concentration as in the 1968 experiments, which could be explained with the higher larval instars present in the population (L_6-L_7) larvae. The effectivity was considerably increased with the Ditrifon combination.

In the fourth year of the field experiments (1970) besides Thuricide also the preparation Biotrol was tried alone (0.2%) and combined with 0.02% Pirimor, Diasinon and Imidan. The results are shown in Table 6 and Fig. 4; the instars of the larval population are shown in Fig. 5.

The effectivity of Thuricide and Biotrol was similar in 0.3% concentration; the low-dosage insecticide combinations increased considerably the effectivity, as seen from the higher initial effects.

Discussion

a) The method based on the reduction of numbers of *B. thuringiensis* colonies proved to be useful in testing pesticides for inhibitory effect. It was stated that *B. thuringiensis* suspensions are compatible with the majority of insecticides used in plant protection. There was however a noticeable damage on spore viability if the insecticide concentrations were too high or the combination ("spray liquid") was allowed to stay for a longer time (24–48 hours). Contrary to insecticides, some fungicides (coppersulphate, Dexon, Karathane, Difolathan, Chlorbenzilat) slowed down or decreased the effectivity *B. thuringiensis*, so the combinations with fungicides should be avoided in the plant protection practice.

b) The field experiments showed also not only the compatibility of many types of insecticides with *B. thuringiensis* preparations but also the possibility of increasing the effectivity of the latter by using them in combinations. The combinations contained in our experiments usually one-tenth of the insecticide concentrations used in the practice, so their use does not seem hazardous neither from point of view of insecticide residues, neither for the beneficial fauna. Most organophosphates tried in the combinations could be recommended for the practice (Diasinon, Ditrifon, Gusathion, Imidan, Lebaycid, malathion, Nogos, phosphamidon, Ultracid).

d) The separate use of *B. thuringiensis* and insecticides did not prove to be superior to combinations. Originally it was intended to facilitate the uptake of *B. thuringiensis* spores, then weakening the larvae by an insecticide. The increased effect could be achieved however also in the mixtures, without the additional expenses of double treatment.

*

The author owes sincere thanks to Professor Dr. Y. TANADA (Berkeley, Calif.) for his guidance and help in the laboratory experiments; the author is also very indebted for the helpful assistance in the field experiments to Dr. E. NAGY (Plant Protection Service), to Mrs. G. CALDERBANK and Miss Á. PALOJTAY.

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Appendix

Commercial names and chemical names of preparations used in combinations with *B. thuringiensis:*

CAPTAN = N-(tricloromethylmercapto)-tetrahydrophtalimide
DL-40 = DDT-lindane (gamma BHC) combination (40 + 4%)
DDT = diclorodiphenyl-tricloroethane
DEXON = sodium-n-dimethylaminobenzene-diazosulphonate
DIASINON = o-o-diethyl-o-(2-isopropyl-4-methyl-pyrimidyl-6)-tiophosphate
DIBROM = o-o-dimethyl-o-2,2-dicloro-1,2-dibromoethylphosphate
DIFOLATHAN = N-1,1,2,2-tetracloroethyl-tiotetrahydrophtalimide
DITRIFON = o,o-dimethyl-1,2,2-tricloro-1-oxy-ethylphosphonate
GUSATHION = o,o-dimethyl-S-(3,4-dihydro)-4-oxo-1,2,3-benzotriazine-3-methyl)-ditiophosphate
IMIDAN = o,o-dimethyl-S-(phtalimido-methyl)-ditiophosphate
KARATHANE = 2-(1-methylheptyl)-4,6-dinitrophenyl-crotonate
LEBAYCID = o,o-dimethyl-o-(4-methylmercapto-3-methylphenyl)-tiophosphate

MALATHION = o,o-dimethyl-S-1,2-dicarbethoxy-ethylditiophosphate
MANEB = manganese-ethylen-bisditiocarbamate
NOGOS = o,o-dimethyl-o-(4-methylmercapto-3-methyl-phenyl)-tiophosphate
PANOGEN = mercuro-methyl-dicyan-diamide
PARATHION = para-nitrophenyl-diethyl-tiophosphate
PHOSPHAMIDON = o,o-dimethyl-o-(2-cloro-2-N-N-diethylcarbamoyl-1-methylvinyl)-phosphate
PIRIMOR = 5,6-dimethyl-2-dimethylamino-4-pyrimidinyl-dimethylcarbamate
SEVIN = 1-naphtyl-N-methylcarbamate
ULTRACID = o,o-dimethyl-S-(2-methoxy-1,3,4-tiodiazol-5(4H)-onyl-4-methyl)-ditiophosphate

Systemic Fungicides and their Mechanism of Action

Acta Phytopathologica Academiae Scientiarum Hungaricae, Vol. 6 (1—4), pp. 311—327 (1971)

Differentiation between Transportable Fungicides and Systemic Infection-inhibiting Agents, and their Relative Perspectives

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This is an introductory paper to a round table discussion at the Conference on Biochemical and Ecological Aspects of Plant-Parasite Relations, held in Budapest, 1970.

I. Introduction

Fungicides aim at killing at places and under circumstances where fungi damage the interests of man. Agricultural fungicides have been developed for the safeguard of man's crops and other plants he deems useful for his purposes against fungal attack. All present-day agricultural fungicides do what their name implies: they kill fungi which damage plants. Nevertheless in agriculture our real goal is not to kill fungi but merely to protect higher plants. Under the impression of the destructive violence of fungal plant diseases we sometimes tend to forget that among higher plants not resistance but susceptibility is the exception. From the fact that among the around 100.000 classified fungal species no more than about 200 are known to cause serious plant diseases, one has to conclude that in nature all higher plants have very effective mechanisms at their disposal to protect themselves. Or, alternatively, that only very few fungal species dispose of successful mechanisms of attack. Anyhow, one may say that in practice every plant species is immune towards the attack of all but a very few species of fungi which are highly specific for the plant species in question and frequently even for particular varieties. In other words, one has to accept a high mutual host/parasite specificity, which modern research has been able to reduce to genetically-defined factors both in the fungus and in the plant. Apart from certain external factors such as humidity, temperature, thickness of the cuticula etc., this implies that the successful attack of a fungus on a plant is dependent on the establishment of a very specific biochemical interrelation between the two organisms concerned. It is possible to disturb this interrelation by killing or at least inhibiting either of the two partners and for obvious reasons we select the fungus to endure this fate. As already said, this job is being done for us by fungicides.

It should be realized that only in very few cases natural resistance is based upon the presence in the plant of fungitoxic compounds. However, as will be dis-

cussed later on, in several cases the penetrating fungus sets going a sequence of processes which result in the topical formation of fungitoxic agents from non-active precursors available in the plant.

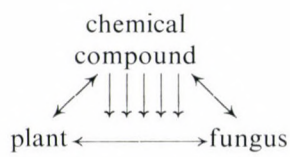
In the past a variety of quite successful agricultural fungicides have been developed. A few incidental cases excepted, they all have in common that the fungus is attacked before it enters the plant. This means that until quite recently far more success had been achieved in protecting plants than in curing them. I am not going to review these *protectant* fungicides since they are well-known to all of us. Suffice it to say, that these "classical" fungicides for many years have fulfilled a splendid job in crop protection and are likely to do so for many years to come.

Around 1940 the idea was conceived to search for fungicidal compounds which can enter the plant and protect it from within, the *systemic* fungicides. Originally HORSFALL and his group followed up this idea for the combat of vascular diseases – in particular Dutch elm disease – which by definition cannot be fought by means of protectant fungicides once penetration of the fungus has occurred. Later on the idea was extended to include two further practically important goals. In the first place the development of *curative* agents which are active not only in the pre- but also, to a limited extent, in the post-penetration stage. Such curative agents are of importance in fighting certain foliar diseases, e.g. the use of organomercurials against apple scab, and in particular for the combat of deep-seated infections in seeds. Systemic compounds of this type should act quickly and need not have a long life-time once within the plant. Secondly, because of the large number of protectant sprays required during the growing season it was hoped that true systemic fungicides could be developed which lend prolonged internal protection once they have been applied to and taken up by the plants. This requires compounds which after being taken up are stable, either as such or in the form of active biochemical transformation products, and thus remain active within the plant for a long period of time. During the past few years considerable progress has been made towards this goal. After a long period of modest progress all of a sudden and almost simultaneously at least five or six remarkably effective types of systemic fungicides have bloomed into existence. These compounds vary between extreme and rather restricted chemical stability, and they range from highly specific to broad spectrum systemic fungicides. Moreover, they exert a surprisingly low mammalian toxicity and negligible phytotoxicity at the required concentrations. With the probability of even more such compounds to come it would seem that the combat of fungal plant diseases, after many decades of placid progress, has now entered an entirely new era. I shall discuss these recent developments later on in my paper.

The search for systemic fungicides has had another, maybe equally important, consequence. One line of approach towards systemic *fungicides* has been the search for systemic *compounds* in general, i.e. compounds which have the innate property to be taken up by the intact plant – either through the foliage or through the roots – and which are transported from the penetration places to other parts of the plant.

During such work it was observed that a number of quite divergent types of systemic compounds—such as certain plant growth regulators, special amino acids and in particular the compound phenylthiourea—exercized a favourable influence upon several fungal plant diseases. Nevertheless, in the usual tests they showed scarcely any fungitoxicity, nor were they converted within the plant into fungitoxic compounds.

Some moments ago I emphasized that the successful attack of a fungus on a higher plant involves the establishment of a specific biochemical relation between the two species, which can be disturbed by killing the fungus. In principle it is possible, however, to disturb this relation in a much more subtle way. Before specifying this further it should be pointed out that chemical compounds when brought under suitable conditions are subject to chemical modification. In fact, studies of the working mechanisms of biocides have shown that in many cases not the original compound, but rather a conversion product is the really active molecule. To illustrate this point I present a diagram, indicating the triangular relation chemical compound/fungus/plant:



It is evident that especially systemic compounds are not only vulnerable to purely physical or chemical modification, but also to biochemical transformation by the living systems, since they operate by definition within the plants. The diagram intends to illustrate how chemical compounds and their chemical and biochemical conversion products may interfere with the relation plant/fungus, not only by a direct influence on either of the two organisms concerned, but as well by disturbing in several ways the specific biochemical interrelation between the metabolic systems of these organisms. It is clear that this approach presupposes an intimate knowledge of the biochemical details of the host/parasite relation, as well as of the defence mechanisms of plants and the offence mechanisms of fungi. Such knowledge is at present still very restricted.

As an example, the two following kinds of interference of chemical compounds with the host/parasite relation can be indicated:

1. The basic metabolism of the plant may be altered such that the plant becomes an unattractive host for the invading parasite. For instance, it has been found that certain types of plant growth regulators cause the sugar level of foliage to decrease. This decrease correlates in a straight-forward way with a diminished or an enhanced resistance in certain host/parasite combinations. The phenomenon clearly must be linked to the distinction, made by HORSFALL and DIMOND in 1957,

between high and low sugar diseases. Another possibility is to look for chemical compounds which interfere with specific receptor places in the plant for toxins excreted by the fungus, but so far no clear cases of such an effect are known.

2. Chemical compounds may inactivate the biochemical weapons of attack excreted by many fungi, such as toxins and tissue-destructing enzymes. In many vascular diseases, e.g. the Dutch elm disease, fungal toxins induce gum formation in the vessels, resulting in a disturbance of water and nutrient transportation. Only one convincing example of a toxin-inactivating agent has been reported, viz. diamino-azobenzene, which was found by HOWARD in 1941 to inactivate the toxin produced by *Phytophthora cactorum*. Many fungi produce extracellular pectolytic enzymes which cause the hydrolytic breakdown of the constitutional pectins in the cell walls of plants. So far no really effective inactivators of these tissue-destructing enzymes have been found. Nevertheless, results published in 1962 and 1968 by GROSSMANN prove the feasibility of this approach. Following root treatment with a 10^{-3} molar solution of rufianic acid (1, 4-dihydroxyanthraquinone sulfonic acid-2) tomato plants were protected against *Fusarium oxysporum*. In this high concentration rufianic acid is not fungitoxic towards *Fusarium*, but is a strong inhibitor of the pectolytic enzymes produced by this fungus. For practical purposes the required concentration is much too high.

In the majority of cases no such distinct targets can be defined if we want to interfere chemically with the new biochemical *system* resulting from the interaction of the two organisms involved: plant and fungus. Nevertheless this possibility in the long run may offer the most selective approach towards fighting fungal diseases of plants. As a support to this approach we have been working in Utrecht for some years on the biochemical backgrounds of the natural mechanisms of susceptibility and resistance.

The following picture summarizes the ways in which non-fungitoxic systemic compounds may increase the plant's resistance against fungal attack:

systemic compounds increasing the plant's resistance by
- changing the plant's metabolism
- interference with the biochemical relation plant–parasite
- inactivating toxins or tissue-destructing enzymes

It should not discourage us that notwithstanding much efforts the practical successes of the systemic compounds at present are very modest in comparison with those of the new systemic fungicides. After all, the realization of the latter has required almost three decades after the conception of the idea.

After these general considerations I shall survey the present factual situation regarding the systemic compounds which affect resistance and the true systemic fungicides. Thereupon the two lines of approach will be briefly evaluated.

II. The Development of Infection-inhibiting Systemic Compounds

As said in the Introduction certain plant growth regulators and amino-acids show a favourable influence upon several fungal plant diseases. Whereas WAIN and his group in Great-Britain studied in particular the effects of synthetic compounds of the phenoxyacetic acid type, VAN ANDEL at Wageningen directed her attention to the relation between resistance and the level of the natural auxin indoleacetic acid (IAA). She found that the susceptibility of cucumber for *Cladosporium cucumerinum* rises and falls with the IAA-level within the plant. Thus, compounds which cause an increase in the IAA-oxidase activity within the plants increase their resistance. Such an effect is shown by certain amino-acids, in particular by L-threo-β-phenylserine (Fig. 1).

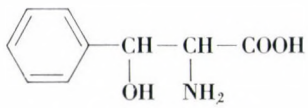


Fig. 1. Phenylserine

A definite explanation for these effects is not yet known, but the possibility to increase the resistance of plants by influencing the level of natural growth factors is certainly a very challenging one.

One of the most effective non-fungitoxic infection-inhibiting systemic compounds found so far is phenylthiourea (Fig. 2)

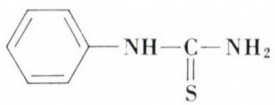


Fig. 2. Phenylthiourea

At Utrecht the mode of action of phenylthiourea has been studied extensively by Dr. KAARS SIJPESTEIJN and her group. A brief discussion of this work may serve to illustrate this line of approach.

Cucumber seedlings when placed for two days with their roots in a 50 ppm solution of phenylthiourea (PTU) were completely protected against the subsequent infection with spores of *Cladosporium cucumerinum*. A similar protective effect of PTU had been observed by KUĆ for the combination apple/*Venturia inaequalis*. The most intriguing aspect of the PTU action is its lack of in vitro fungitoxicity. At least 500 ppm of PTU are required to inhibit growth of *Cl. cucumerinum in vitro*. On the other hand it was found that in press sap of protected cucumber plants the PTU content did not exceed 10—20 ppm. The press sap did

not contain any fungitoxic compound which might have been formed by chemical transformation of PTU. Neither did it show any inhibitory effect against the pectolytic enzymes excreted by *Cl. cucumerinum*. Thus, all evidence indicated that we are dealing here with a systemic compound which either influences plant metabolism, or interferes directly with the biochemical relation between fungus and plant.

Now it was known already that PTU is a strong inhibitor of polyphenoloxidases. Using a simple polyphenoloxidase inhibition test it could be shown at Utrecht that the protecting capacity of a number of structural variants of PTU towards the cucumber/*Cl. cucumerinum* combination parallels their polyphenoloxidase-inhibiting activity, PTU itself being the most active compound in both respects.

A second effect of PTU, observed by KAARS SIJPESTEIJN and SISLER (1968), was the increased peroxidase activity it incited in treated cucumber plants. It is well-known that peroxidases play an important part in the biosynthesis of lignin from phenolic plant constituents. In 1963 HIJWEGEN, at Wageningen, observed that resistant cucumber varieties upon inoculation with *Cl. cucumerinum* respond with considerable lignification around the infection places. Much less lignification was induced upon inoculating susceptible varieties. He postulated that the much stronger lignification in the resistant plant puts up efficient barriers for the invading fungus. Now it is very suggestive that susceptible cucumber varieties which as a result of PTU-treatment have become artificially resistant, react in exactly the same way upon inoculation with *Cl. cucumerinum* as naturally resistant varieties do, viz. with strong local lignification at the penetration points.

It is tempting to suggest that the enhanced lignification as a result of PTU-treatment is not only caused by an increased peroxidase activity but is as well a consequence of the availability of larger amounts of phenolic lignin precursors, due to the inactivation of polyphenoloxidases.

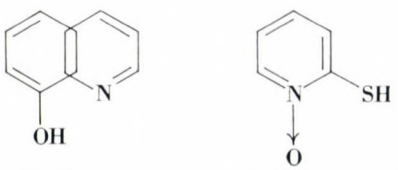
Meanwhile, one should not carry through too far this suggestive comparison between the reactions of naturally and artificially resistant plants. For instance, in the so-called hypersensitivity reaction a totally different mechanism of natural resistance is operative. An interesting case is the resistance and susceptibility of apple to *Venturia inaequalis*, studied at Utrecht by RAA and OVEREEM. All apple varieties store in their leaves enormous quantities of a non-fungitoxic glucoside, phloridzin. It was found that in resistant apple plants *V. inaequalis* is killed by a fungitoxic ortho-quinone formed upon oxidation of phloridzin. This quinone is not formed in susceptible plants. The explanation of this different behaviour lies in the nature of certain toxins excreted by *V. inaequalis*. The remarkable thing is that these fungal excretions are toxic to cells of resistant plants and non-toxic to those of susceptible ones. The effect is that leaf cells adjacent to the infection places are damaged in the resistant plants and this sets going a sequence of oxidation reactions resulting in the formation of the fungitoxic quinone mentioned before. Thus, as a consequence of their reaction upon a fungal toxin, in resistant

plants a latent defence mechanism is triggered off which kills the invading fungus, whereas in susceptible plants no such reaction occurs and the fungus can grow unhampered.

It should be obvious that the search for infection-inhibiting systemic compounds is impeded by several factors. First, only plant/parasite *combinations* can serve as test objects, but this is mainly a technical matter. Secondly, and much more serious, no really logical lines of approach can be defined since too little is yet known about the biochemistry of natural resistance and susceptibility. But even if one has obtained some insight into this biochemistry, as is e.g. the case for the combination apple/*Venturia inaequalis*, it is still a matter of uncertainty how to make use of it. Here, as is true for discovering new fungicides—both protectant and systemic—, one has to hit upon the right observations which is even now, after many decades of intensive research, still much dependent on trial and error.

III. The Development of Systemic Fungicides

The study by HORSFALL and ZENTMYER at the Connecticut Agricultural Research Station of 8-hydroquinoline as a systemic chemotherapeutant against Dutch elm disease stand as a landmark at the beginning of this line of thinking. A similar active compound with strongly chelating properties, 2-mercaptopyridine-N-oxide, (Fig. 3) was studied by SANDER and ALLISON (1956) and by ROMBOUTS and KAARS SIJPESTEIJN (1958).



8–Hydroxyquinoline 2–Mercaptopyridine–N–oxide

Fig. 3

Although both compounds and certain derivatives were quite effective under experimental conditions, they did not succeed in the field.

In relation to other work with amino acids VAN ANDEL in 1962 made the interesting observation that ortho-, meta- and in particular parafluorophenylalanine act systemically in the protection of cucumber against *Cladosporium cucumerinum* and *Colletotrichum lagenarium* (Fig. 4).

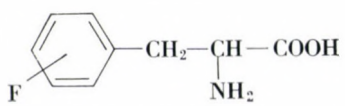


Fig. 4. *o*, *m* and *p*–Fluorophenylalanine

All three compounds are active in vitro fungicides but are counteracted by phenylalanine. They are likely to act as competitive antagonists of this natural amino acid.

A closely related compound was recognized in the early sixties by KASLANDER and DEKHUIJZEN at Utrecht as an enzymatic conversion product of dimethyldithiocarbamates, the conjugate DDC-alanine (Fig. 5).

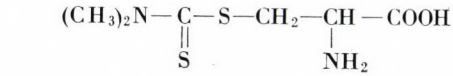
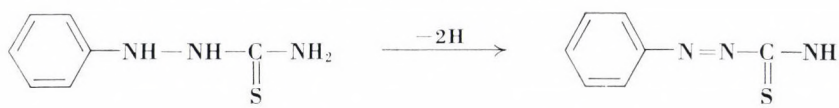


Fig. 5. DDC-alanine

It is easily taken up by and translocated within plants, rather stable within the plant's system and not very phytotoxic. On the other hand it is a powerful fungicide because in vitro fungal mycelium rapidly converts the conjugate into the parent fungicide, the dimethyldithiocarbamate anion. At that time we really believed to have discovered an almost ideal type of systemic fungicide, until we found out that DDC-alanine had a deceptively low activity against plant/parasite *combinations*. A closer study revealed that two factors strongly decrease the effectiveness of DDC-alanine as a systemic fungicide. First, several aliphatic amino acids present in the plant, e.g. alanine, counteract the formation of the fungitoxic DDC-anion from DDC-alanine. Secondly, the plant contains effective antagonists of the DDC-anion as a fungitoxic agent, e.g. histidine and, maybe, still other strongly chelating agents. End of a dream!

In the same period PLUIJGERS at Utrecht discovered the direct and systemic fungicidal activity of 1-phenylthiosemicarbazide. This compound is taken up both by the roots and the foliage and is translocated as such within the plant. It appeared that actually not the compound itself but a dehydrogenation product, phenylazothioformamide, is the active molecule (Fig. 6).



1-Phenylthiosemicarbazide Phenylazothioformamide

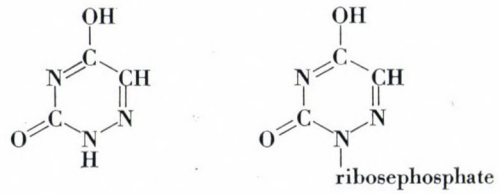
Fig. 6

Although both compounds are effective against quite a number of host/parasite combinations, again the performance in the field was disappointing.

Quite a different approach has been the evaluation of a number of fungitoxic antibiotics. Remarkably enough, most antibiotics are more or less systemic. Since BRIAN's first favourable reports in 1952 on griseofulvin several other antibiotics have been shown to be effective systemic fungicides: e.g. cycloheximide,

streptomycin and in particular rimocidin and pimaricin. Notwithstanding some remarkable successes, e.g. the practical application in Japan of blasticidin against rice blast, I do personally not believe in the large scale development of any antibiotic as an agricultural fungicide. Their production from natural sources is very expensive and their chemical structures are so complicated that an economic synthesis, at least within agricultural price levels, seems quite impossible.

A more or less isolated case was the important observation of DEKKER at Wageningen in 1964 that the purine analogue 6-azauracil shows considerable



6–Azauracil 6–Azauridinephosphate

Fig. 7

systemic activity against mildew on various plants. This compound, in the form of its biochemical transformation product azauridinephosphate, interferes with purine metabolism, in particular in the fast-growing mould (Fig. 7).

In spite of all these efforts and partial successes, until six years ago it had to be admitted that so far no really useful systemic fungicides had been developed.

Since 1964, however, a perplexing variety of notoriously successful systemic fungicides has appeared in quick succession on the agricultural scene. They belong to quite different chemical classes, but have in common that they are all substituted heterocycles. Now, fungicides containing heterocyclic ring systems were certainly not new. Glyodin, 2-heptadecyl-2-imidazoline acetate, discovered in 1946 at the Boyce Thompson Institute has found extensive use as a foliage fungicide. In fact, the first practically applied systemic fungicide was a heterocyclic compound as well. It is the compound "Wepsyn 155", 1-bis-(dimethylamino)-phosphoryl-5-phenylamino-1,2,4-triazole, described in 1960 by VAN DEN BOS et al. at Philips-Duphar. Wepsyn and a few closely related compounds are systemically active against powdery mildews and against apple mildew (Fig. 8).

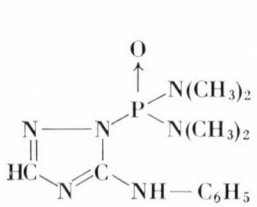


Fig. 8. Wepsyn 155

Prominent among the new systemic fungicides are two derivatives of benzimidazole, thiabendazole and benomyl. Both are active against a wide range of fungal pathogens. Thiabendazole, 2-(4'-thiazolyl)benzimidazole had originally been developed by Merck & Co as an anthelmintic. In 1964 STARON and ALLARD described its systemic fungitoxicity for the first time (Fig. 9).

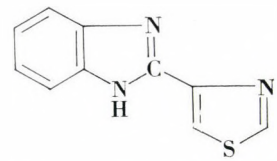


Fig. 9. Thiabendazole

The systemic activity of the at present most striking fungicide benomyl, 1-(butylcarbamoyl)-2-benzimidazole carbamic acid methyl ester, was reported first by DELP and KLÖPPING in 1968 (Du Pont de Nemours) (Fig. 10).

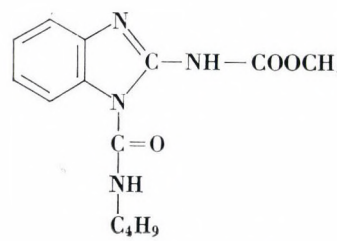
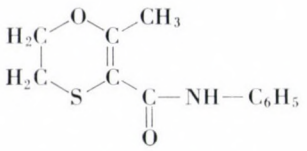


Fig. 10. Benomyl

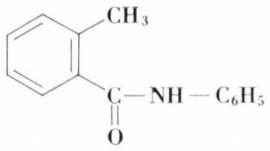
Among all the new systemic fungicides this is certainly the one with the most noteworthy properties. It is highly active against a wide range of foliage diseases, including powdery mildews and also against many soil-borne pathogens. Neither thiabendazole nor benomyl is active against phycomycetes, e.g. *Phytophthora infestans* (potato blight), *Plasmopara viticola* (vine downy mildew) and *Pythium spp.* In addition, benomyl has shown to exert a mite ovicidal action. A few things more will be said about benomyl in some moments. First I shall survey briefly the other new types of systemic fungicides.

In 1966 von SCHMELLING and KULKA of Uniroyal announced the development of carboxin, 2.3-dihydro-5-carboxyanilido-6-methyl-1,4-oxathiin, the effects of which were described by EDGINGTON et al. in the same year (Fig. 11).

Carboxin (or "vitavax") is particularly effective against deepseated basidiomycetic infections of seeds (e.g. smut in wheat and barley). The corresponding sulphone ("plantvax") has a slightly different biological spectrum. The structure of carboxin is somewhat related to that of mebenil, 2-methylbenzanilide, developed by BASF, which is effective against rusts on cereals.

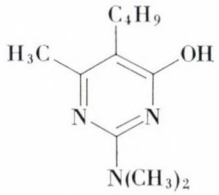


Carboxin ("vitavax")

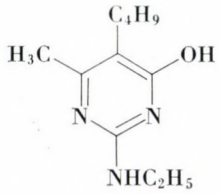


Mebenil

Fig 11



Dimethirimol ("milcurb")



Ethirimol ("milstem")

Fig. 12

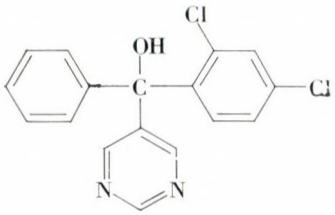


Fig. 13. EL–273

Two pyrimidine derivatives dimethirimol ("milcurb") and ethirimol ("milstem") announced by ICI in 1968 are specifically effective as a soil treatment for the control of powdery mildews (Fig. 12).

Both compounds are fairly stable in soil, are rapidly taken up and translocated but do not accumulate, since they are broken down, or at least inactivated, within a few days in the plant.

Another pyrimidine derivative is the broad spectrum systemic fungicide EL-273, developed by Eli Lilly & Cy. It is α-(2,4-dichlorophenyl)-α-phenyl-5-pyrimidinemethanol (Fig. 13).

A number of further promising systemic fungicides I can only mention in passing (Fig. 14).

The last mentioned type of fungicide, the thiophanates, seems to occupy an exceptional position since it is no heterocyclic compound, but instead a disub-

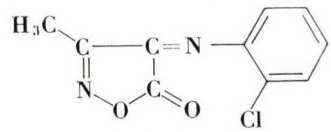
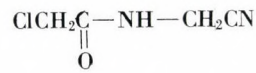
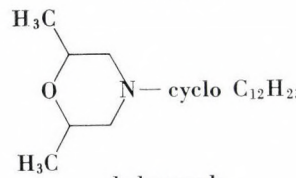
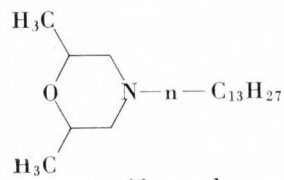
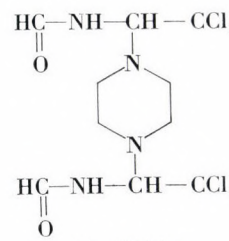
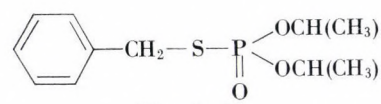
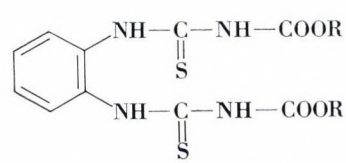
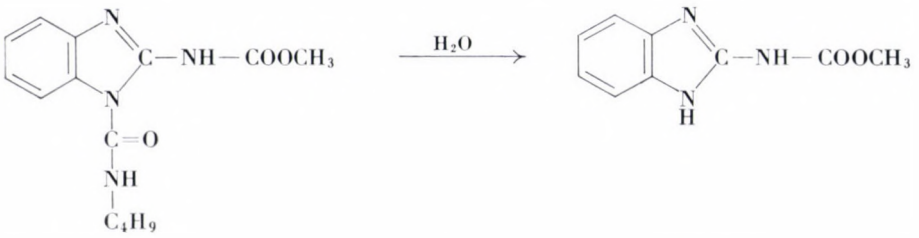
Developed by	*Structure*	*Diseases*
Plant Protection	 drazoxlone	mildew
Nippon Soda	 Udonkor	powdery mildew
BASF	 dodemorph	mildew
BASF	 tridemorph	mildew
Boehringer/Cela	 Cela W 524	powdery mildew, apple scab
Kumiai	 Kitazin P	rice blast
Nippon Soda	 R = CH_3 or C_2H_5 the thiophanates	broad spectrum, like benomyl

Fig. 14. New systemic fungicides



Benomyl BCM

Fig. 15

stituted benzene derivative. AELBERS, at the Symposium for Phytopharmacy and Phytiatry at Gand earlier this year, related the development of these bisthioureidobenzene derivatives to the discovery in 1962 by KAARS SIJPESTEIJN and PLUIJGERS of the systemic protectant action of phenylthiourea. It was found that both methyl and ethyl thiophanate are broad spectrum fungicides which have the same antifungal spectrum as benomyl, be it on a somewhat lower level of activity.

In 1969 it was reported by CLEMONS and SISLER in the USA that benomyl is unstable in aqueous systems. The butylcarbamoyl group is quickly split off by hydrolysis with formation of benzimidazole carbamic acid methylester (BCM) (Fig. 15).

Both the antifungal spectrum and the level of fungitoxicity of benomyl and BCM are identical. Following treatment with benomyl the only fungitoxic agent found within plants is BCM and it must, therefore, be accepted that the systemic action of benomyl is entirely due to BCM. The latter compound is extremely stable both in vitro and within plants. Until now it is completely unclear what the function is of the butylcarbamoyl group in benomyl, since it is already removed under the mildest conditions. It certainly does not play a role in the systemic action of benomyl. The only thing to suggest at present is that the butylcarbamoyl group may serve as an inbuild formulation factor, preventing perhaps too quick an absorption of the fungicide by the plant.

It is interesting to note that the parent compound 2-aminobenzimidazole (Fig. 16)

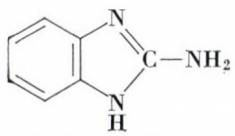


Fig. 16. 2-Aminobenzimidazole

is devoid of any fungicidal or miticidal activity but is, contrary to benomyl and BCM, rather phytotoxic.

I now return to the thiophanates. Taking into account the identity of the antifungal spectra of benomyl and BCM with those of the thiophanates, it occur-

red to Dr. KAARS SIJPESTEIJN at Utrecht that the following chemical conversion should be considered (Fig. 17):

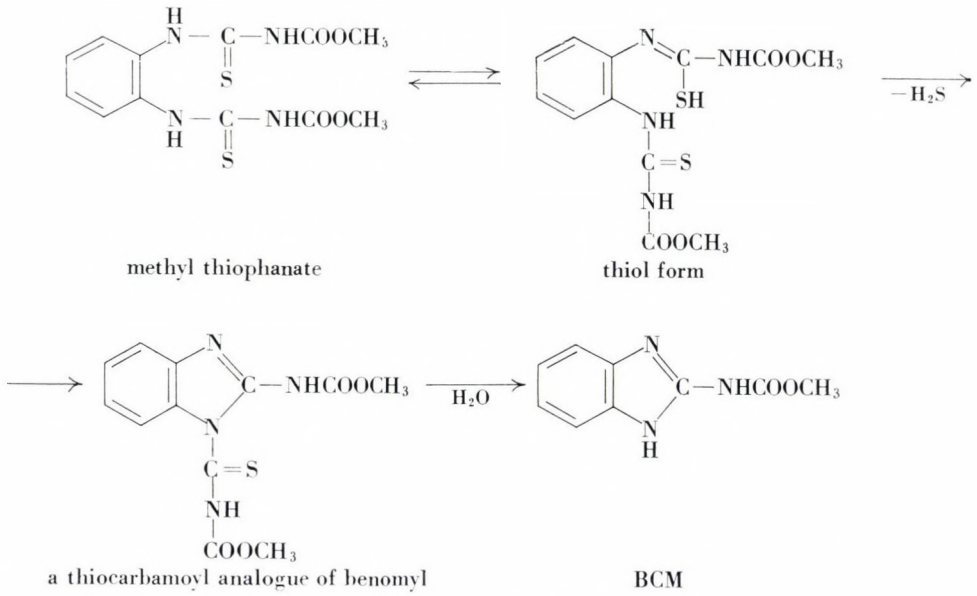


Fig. 17

At our Institute VONK and SELLING could indeed demonstrate the formation of BCM from methyl thiophanate under mild chemical conditions, as well as that of the ethyl analogue of BCM from ethyl thiophanate. The conversion is much accelerated in the presence of the ions of certain heavy metals, e.g. Cu^{2+}. These results strongly suggest that the fungitoxicity as well as the systemic action of methyl and ethyl thiophanate may at least in part be attributed to their easy transformation into BCM and its ethyl analogue respectively.

Little is known yet about the biochemical modes of action of the several new fungicides. At Utrecht Dr. KAARS SIJPESTEIJN has analysed the general principles underlying the biochemical modes of action of the present-day agricultural fungicides, both protectant and systemic. She came to the remarkable conclusion that quite generally those fungicides which interfere with the *energy-producing processes*, e.g. the inhibitors of cell respiration, do not include any really systemic compound. On the other hand, all systemic fungicides appear to interfere with *biosynthetic processes* and thus in a direct way with growth. The following table, taken from her recent paper "Biochemical modes of action of agricultural fungicides" (World Review of Pest Control, 1970, p. 85), illustrates these points.

She argues that metabolism in higher plants and fungi is basically similar at the enzyme level. Consequently, fungicidal inhibitors of energy-producing

Table 1

Mode of action	Active as systemic fungicides
Inhibitors of energy production	
thiram	−
triphenyltin acetate	−
dinocap	−
nabam	−
captan	−
folpet	−
tetrachloroisophthalonitrile	−
phenylmercuric acetate	−
Inhibitors of biosynthesis	
sulfanilamide	+
cycloheximide	+
carboxin	+
benomyl	+
thiabendazole	+
azauracil	+
griseofulvin	+
chloroneb	+
Disruptors of cell membrane	
dodine	±
polyenes	±

processes are equally effective towards higher plants once they penetrate the plant. This has been nicely demonstrated by Dr. VAN ANDEL at Wageningen a few years ago. She found that insoluble dithiocarbamates, which are very safe protectant fungicides, are taken up by plants if administered in the simultaneous presence of certain amino acids. Thus, under these circumstances the dithiocarbamates become systemic, but VAN ANDEL observed that at the same time they become extremely phytotoxic as well. On the other hand, compounds which, like the true systemic fungicides, interfere with biosynthetic processes are much more active towards the fast growing fungi than against higher plants, which grow much slower and where growth processes are, moreover, very localized.

In the meantime work from several sides has indicated that many of the new systemic fungicides interfere in one way or the other with purine metabolism and thus act at the RNA or DNA level, i.e. they interfere with very specific parts of cell metabolism.

IV. Conclusions

I have been asked to reflect upon the differentiation between transportable fungicides and systemic infection-inhibiting agents, and their relative perspectives. This, finally, requires a critical comparison between the two subjects which I

have reviewed more or less separately. When meditating on this topic it occurred to me that, in fact, there is very little to compare, and that this is true for more than one reason.

In the first place we are dealing here with two completely different lines of thought. To repeat it briefly, systemic fungicides are first of all *fungicides*, i.e. they disturb the relation plant/fungus exclusively by killing or at least inhibiting the fungal parasite. On the other hand, infection-inhibiting systemic compounds interfere with the defence mechanisms of plants, with the offence mechanisms of fungi or with the biochemical interrelationship between plant and fungus.

In the second place, as I have mentioned before, true systemic fungicides are without exception compounds which interfere with biosynthetic processes and all evidence available at present indicates that they do so in a very specific way, e.g. by interfering with purine metabolism. It is not at all impossible that the shift from the non-specific protectant fungicides, which are general inhibitors of the energy-producing enzyme systems, to the systemic fungicides will be accompanied by a phenomenon which so far has been of minor importance in fungicidal applications, viz. the development of fungicide resistance. DEKKER at Wageningen reported in 1968 on the isolation of mutant strains of *Cladosporium cucumerinum* which are resistant against azauracil. Also the development of resistance against certain fungitoxic antibiotics and, remarkably enough, against organomercurials has been observed.

Until now this alarming phenomenon of the development of resistant fungal strains has never been observed at a practical level for protectant fungicides. Before proceeding to the large scale application of the new systemics the answers to the resistance problem should be known. Among the agricultural chemicals the fungicides so far have enjoyed a reasonably benevolent press. I believe that we are not allowed again, as in the case of the insecticides, to learn our lesson by bitter experience.

As regards the infection—inhibiting systemic compounds it would seem to me that even a very specific interaction with the biochemical interrelationship between plant and fungus makes the potential danger for developing resistance very remote, since such compounds in principle do not act on the fungal species as such.

Regarding this aspect I thus see in the long run distinct advantages for the systemic compounds at least from a fundamental point of view. This latter must be emphasized, since we have to admit that at present the available systemic fungicides are much better performers against a wide range of important plant diseases than the few systemic compounds which have been found so far. As outlined before, following up this line of thought requires the extension of our fundamental knowledge regarding the underlying causes for natural resistance and susceptibility, including the defence mechanisms of plants and the offence mechanisms of parasitic fungi. Such knowledge may become important as well from an entirely different point of view. The agriculturists' dream is, of course, to elim-

inate plant diseases by breeding resistant plant varieties and thus to eliminate the necessity of using fungicides at all. So far the successes towards this goal have been rather modest, not in principle but in practice. The frequent appearance of new races of fungi which are capable of attacking resistant plant varieties has taught us the present uncertainty and incalculability of this approach.

In one important respect the systemic fungicides and the systemic compounds have something in common which deserves our utmost attention. The classical protectant fungicides are intended to do a rather rough and indiscriminative job *outside* the plant in which the latter is involved as little as possible. As a consequence they are chemically quite reactive and mostly not very persistent. They have even been blamed for this quality because too many sprayings are required to protect crops throughout the growing season. This, of course, is not entirely due to chemical instability but as well to the formation of unprotected plant surfaces during the growth process. On the other hand, so far little indication has been obtained for any hazards connected with their chemical degradation products. In summary, the present-day protectant fungicides perform a reasonably good job at apparently tolerable risks. Now, to the contrary, systemics are intended to enter the plant and often to lend prolonged internal protection. Here we consciously introduce the possibility that residues of the chemicals in question may be present in the harvested crops to which the consumer will be exposed. In other words, we are entering a road which for the insecticides and herbicides has invited much – admittedly often unjustified – opposition. In my opinion the practical use of any systemic, whether it be a fungicide or an infection-inhibiting compound, presupposes a *complete* knowledge of its chemical and metabolic fate within the plant. It is not sufficient just to demonstrate that the original compound is no longer there. We have seen, for instance, that benomyl does not enter the plant at all but that BCM is the actually systemic compound. The example I have given regarding the chemical conversion of the thiophanates may teach us how far one actually can get from home. Only if we know the complete fate of systemics are we able to judge the risks involved and to weigh them against the advantages. What I have said has not the intention to make us shy away from systemics, but to emphasize the type of knowledge we need in continuation of having established that they are effective plant protecting agents.

I hope that I have been able to impress upon you how far the modern views on chemical plant protection are already beyond the aims of the classical approach. But let us stay modest. The classical fungicides have done and are still doing an excellent job, also in economic respect. Notwithstanding the present splendid outlooks offered by the new era of systemics, in particular the systemic fungicides, it should be realized that very much remains to be done before we can say that the days of the classical agricultural fungicides are over.

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Problems of Selectivity in the Field of Systemic Fungicides

By

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This is an introductory paper to a round table discussion at the Conference on Biochemical and Ecological Aspects of Plant–Parasite Relations, held in Budapest, 1970.

The possibility of using systemic fungicides for the control of plant diseases has received attention from phytopathologists and chemists since many years. It is almost half a century ago that A. MÜLLER wrote the first book on systemic fungicides: "Innere Therapie der Pflanzen". A more extensive search for systemic compounds started after the last world war in various parts of the world. For a rather long period of time the results obtained in this field of research did not lead to practical application; they were nevertheless of importance for deepening our insight into the mechanism of action of systemic compounds. Only recently several systemic fungicides have become available for use in agriculture and horticulture. This resulted in an increasing interest in these compounds, which was reflected at the 7th International Congress of Plant Protection in Paris (21–25 September 1971), where about 60% of the papers on fungicides dealt with systemic compounds. It seems, therefore, very appropriate that at this symposium special attention is paid to certain aspects of systemic fungicides.

What are systemic fungicides? Strictly speaking, only those compounds which show fungicidal action *in vitro*, and which are transported through the plant system, should be called systemic fungicides. Usually, however, also those fungicides are included, which do penetrate into the plant tissue, but show little transport; for these the somewhat paradoxal expression "locally systemic" is often used. Also included are systemic compounds which exert no fungicidal action *in vitro*, but which still may control fungal plant diseases, for example by increasing the resistance of the host plant. For this reason the word "systemics" instead of systemic fungicides may be used.

One aspect, which is of special importance in the study of systemic compounds, is the problem of *selectivity*. With respect to fungicides the word selectivity may be used in two ways. It may relate to the biological spectrum of activity or to the biochemical mechanism of action.

Let us first consider the biological spectrum of activity. Each fungicide, which is used for plant protection, has to be selective in its action with respect to host plant and parasite: it should kill the fungus or inhibit its development, but not be harmful to the plant. Our commonly used agricultural fungicides owe their selectivity to nonpenetration into the epidermal cells of the host, which are covered by the cuticle. In principle, however, they are rather non-specific plasma toxicants. For systemic fungicides, which come into contact with both the protoplasm of the fungus and the host cell, the situation is quite different. Obviously, in this case selectivity requirements are very high.

Systemics may also show selectivity with respect to fungicidal action. The spectrum of activity may vary widely for various compounds; some are active against only one particular fungus or group of fungi, others inhibit a wide range of fungi. The spectrum of activity with respect to diseases is for a large part determined by the fungicidal spectrum of the systemic compound and the occurrence of toxicity to different plants. These factors, however, are not the only determinants. Instability of the fungicide in the plant tissue or adsorption to plant constituents before the site of action is reached, may reduce disease control. These factors, as well as uptake and transport of the fungicide, may vary for different plants, and may play a role in the selective action of a systemic. Indirect action of a systemic against a disease, e.g. enhancement of disease resistance, depends on interference of the compound with the metabolism of the host or the host–parasite complex, which may be different for various combinations.

When a fungicide acts on one or only a few steps in the metabolism of the fungus, in contrast to general enzyme- or protoplasm poisons, I should like to call this *biochemical selectivity*. Although there may be a relation with biological selectivity, it is not the same. A compound with a biochemically selective action may show little *biological selectivity*, when the inhibited enzymatic reaction is important both for host and parasite. On the other hand a compound with little biochemical selectivity, may still be selective in its action as a consequence of differences in permeability or in detoxification mechanisms of the host and parasite.

As the mechanism of action of most systemic fungicides is still largely unknown, it is difficult to understand the basic principles underlying the selective action of these compounds. Therefore, considerations about this topic have still a rather speculative character, and examples of selective action to be discussed will for a large part be chosen from results obtained with antibiotics and other antimetabolites. Although many of these, for various reasons, can not be expected to become of practical value, they still may be of great help to us in understanding the mechanism of selective action.

After mentioning a few examples of selective systemic fungicides, we shall first focus our attention on the possible mechanisms involved. Thereafter we will consider some possible disadvantages of the use of highly selective fungicides.

Examples of Selective Action

Already many thousands of chemicals have been screened for selective systemic action against plant diseases. It is encouraging that, especially in recent years, various compounds have been developed, which combine a strong effect on the parasite with no or only slight side effects on the plant. Outstanding among these is certainly *benomyl* 1-(butylcarbamoyl)-2-benzimidazole-carbamate, which is active against a wide range of diseases, which were hitherto difficult to control. In our laboratory BOLLEN and FUCHS investigated its fungicidal spectrum *in vitro*. They observed a remarkable correlation between sensitivity to *benomyl* and taxonomic position, especially with respect to sporogenesis. Entirely to comparatively resistant were fungi belonging the *Oomycetes* and *Zygomycetes*, and among the *Moniliales* those fungi which are characterized by producing porospores or, with a few exceptions, annellospores. Sensitive to very sensitive were fungi belonging to the *Ascomycetes*, to the *Sphaeropsidales* and among the *Moniliales* those fungi which are characterized by forming blastospores, phialospores and aleuriospores. Evidence has been obtained that a similar relation exists for some thiophanates. Some *oxathiins* (Plantvax, Vitavax) are active against fungi belonging to the *Basidiomycetes*, but inactive against other fungi. Various *pyrimidine derivatives* are especially active against powdery mildew fungi, such as 6-azauracil, as has been discovered at Wageningen in 1961, and ethirimol and dimethirimol recently developed in England. At our laboratory the mechanism of action of the anti-mildew compound Hoe 2873, which is 2-(O,O-diethyl-thionofosforyl)-5-methyl-6-carbethoxypyrazolo-(1,5a)-pyrimidine is under investigation by DE WAARD, and the systemic action against powdery mildews of W 524, which is N,N'-bis-(1-formamido-2,2,2-thrichloroethyl)-piperazine by FUCHS.

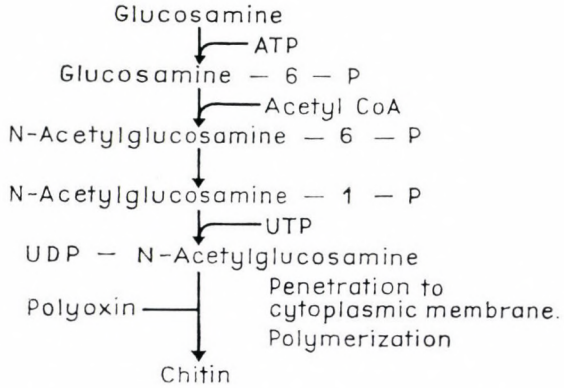
The number of new systemic fungicides with selective action is still increasing. Research on these compounds is going on in various countries. It may be expected that in the near future important information will become available concerning the principles, on which selective action is based.

Selective Action on the Host—Parasite Combination

Each systemic fungicide should be selective with respect to the host—parasite combination since otherwise its use as a plant protectant would be prohibited. Selective action may depend upon:

1. *Interference with a metabolic process which operates in the fungus, but not or not to the same extent in the plant.* The classic example is chitin synthesis, which is important for the formation of the cell wall in most fungi, but absent in plants. Recently, a fungicidal antibiotic named *polyoxin* has been discovered in Japan, the main action of which seems to be on chitin synthesis (SASAKI et al., 1968). It is produced by *Streptomyces cacaoi* var. *asoensis*, a soil actinomycete.

It is a mixture of closely related compounds, 12 of which have been isolated and chemically characterized. One of these, polyoxin D is effective against rice sheath blight, caused by the fungus *Cochliobolus miyabeanus*. In vitro this fungus is inhibited by a concentration of 15 ppm of this antibiotic, whereas a 800 ppm spray does not cause damage to rice plants. Some other fungi, such as *Pellicularia sasakii* and *Alternaria kikuchiana* are also inhibited, but bacteria and yeasts are insensitive. It appeared that polyoxin D interfered with the incorporation of glucosamine into chitin (Fig. 1).



It has been pointed out by ISONO et al. (1969) that there is a gross structural similarity between polyoxin D and uridine diphosphate N-acetylglucosamine, which is believed to be the active form of N-acetylglucosamine in chitin synthesis.

2. *Interference with the same metabolic process both in host and parasite.* In this case a selective action may still be possible due to other factors, such as a stronger accumulation of the fungicide in the fungal cells than in those of the host plant. Another possibility is a difference in growth rate between the fungus and the plant tissue at the site of infection. The action of 6-azauracil (AzU) against cucumber powdery mildew probably is an example of this phenomenon. The cucumber plant is relatively insensitive to concentrations of AzU, which check growth of the pathogen completely. It is known that AzU, after conversion to the nucleotide 6-azauridine-5'-phosphate, interferes with pyrimidine biosynthesis by inhibition of the enzyme orotidine-5'-phosphate (OMP) decarboxylase. During and shortly after infection the fungus is in a phase of rapid growth in contrast to the infected host cell. As rapid growth implies rapid synthesis of nucleic acids, it is conceivable that a compound which interferes with pyrimidine synthesis, and therefore with formation of RNA, is more toxic to the fungus, than to the host cell. In the selective action of AzU still another factor might be involved. From experiments with radioactivity labeled substrate it appeared that the OMP decarboxylase activity in cucumber tissue was at least a hundred times as high as that in a comparable extract of mycelium of *Cladosporium cucumerinum*, the cause of

cucumber scab. As a consequence, inhibition of plant growth in this case requires more of the toxicant than inhibition of fungal growth, so that a dosis AzUMP can be chosen which checks fungal growth, but at the same time interferes little with the metabolism of the invaded host cell (DEKKER, 1968). It is, however, difficult to avoid the risk of phytotoxicity under all circumstances, when the action of the fungicide is at the same site both in plant and parasite. So, repeated spraying of AzU against apple powdery mildew in an orchard gave excellent control of the disease, but also caused malformations of young shoots, plant parts in which rapid RNA synthesis occurs.

3. *Indirect action of a compound which shows no fungicidal activity in vitro.* In this case we may deal with an increase in host resistance. An example of this kind is the action of phenylthiourea (PTU) against cucumber scab (VAN DER KERK, 1969). PTU is in vitro almost inactive against *Cladosporium cucumerinum*, but protects cucumber plants against scab, caused by this pathogen, when the seedlings prior to inoculation are placed in a 20 ppm solution of this compound for 2 days. It was demonstrated that PTU at low concentration inhibits polyphenoloxidase and enhances peroxidase activity in cucumber plants. It is suggested that the increased resistance is due to enhanced lignification upon infection. The stronger lignification might be caused not only by an increased peroxidase activity, but also as a consequence of the availability of larger amounts of phenolic precursors, due to inactivation of the polyphenoloxidase.

Control of a fungal disease by a non-fungicidal systemic might further be caused by inhibition of fungal enzymes, necessary for infection and colonization, or by inactivation of a fungal toxin, responsible for the disease symptoms. No clear-cut examples of these possibilities are available as yet. Of interest is the possibility that plant disease control with a systemic may be achieved via the root exudates, as postulated by STAŇKOVÁ and DEKKER (1970). They observed that soaking cucumber seeds a 1 ppm solution of 6-azauracil protected the roots of the developing seedlings against damping off, caused by the soil pathogen *Pythium debaryanum*, a fungus which *in vitro* is insensitive to 6-azauracil. A 10 hours soak of the seeds in a 1 ppm AzU solution did increase the number of bacteria in the rhizosphere, which are antagonistic to *P. debaryanum;* higher concentrations however, were inactive.

In many cases it will be difficult to explain the selective action of a systemic compound on the combination of host and parasite, due to the complexicity of this system. It is obvious that also differences in uptake, transport, inactivation by the plant tissue etc. may play a role in selective action.

I want to proceed now to a somewhat simpler relation, namely that of a fungicide with a fungus.

Selective Fungicidal Action in vitro

Many systemic fungicides show a highly selective action against fungal and bacterial plant pathogens *in vitro*. Among these are the systemics mentioned in the introduction, and further various antibiotics and other antimetabolites. This selectivity may be due to differences at the site of action in the microorganism or in reaching the site of action by the fungicide.

a) *Reaching the site of action.* The cell wall of a fungus is seldom a barrier to penetration by a fungicide. The protoplasm membrane, however, may show differences in permeability to fungicides.

Blasticidin-S, an antibiotic produced by *Streptomyces griseochromogenes* and used on a large scale for control of rice blast in Japan, inhibits the causal organism of this disease at concentrations of 5 to 10 μg/ml. This antibiotic interferes with protein synthesis. Resistant strains were obtained which tolerated even 1000 – 4000 μg/ml of this antibiotic. As in cell-free extracts from these strains protein synthesis was inhibited by blasticidin-S, it was concluded that permeability probably plays a decisive role in the resistant strains (HUANG et al., 1964).

The fungicide may further fail to reach the site of action in the fungus by rapid detoxification. The resistance of *Fusarium lycopersici* to ascochitin appears to be due to a reduction of this antibiotic by this fungus to the less fungitoxic dihydro derivative (OKU and NAKANISHI, 1964).

b) *Differences at the site of action.* Fungi belonging to the *Oomycetes*, which contain little or no chitin in the cell wall, will probably be less or not sensitive to fungicides which primarily interfere with chitin synthesis, such as polyoxin-D. Differences in sensitivity to a fungicide may also be due to components of the protoplasm membrane. The systemic fungicidal antibiotics rimocidin and pimaricin are active against a wide range of fungi, except the *Oomycetes*, and are inactive against bacteria. These antibiotics cause a leakage of the protoplasm membrane in sensitive fungi, ultimately leading to death. It was shown that this leakage was due to a binding of the fungicide to certain sterols in the protoplasm membrane. Fungi belonging to the *Oomycetes* and bacteria lack these sterols (SCHLOESSER, 1965).

Growth of the fungus *Coprinus lagopus* is inhibited by ethionine. LEWIS (1963) obtained some strains resistant to ethionine. The resistance appeared due to a slight change in the methionine-activating enzyme. In the wild strain this enzyme also activates ethionine, which leads to death of the fungus, but failed to do so in the resistant mutant due to a lack of affinity to ethionine. This was explained as the result of a mutation in the structural gene, which forms the messenger RNA coding for this enzyme.

When a fungicide comes in contact with the protoplasm of the fungal cell, it may or may not interfere with the metabolism of the cell. The antibiotic cycloheximide, which is used as a systemic against various plant diseases, is a powerful fungicide, but inactive against bacteria. In sensitive fungi it inhibits protein syn-

thesis, namely the transfer of aminoacyl-soluble RNA to the ribosomes and the formation of the peptic bond. Although its mechanism of action has thus been elucidated, the selective action on micro-organisms remains unexplained. Some light was thrown on this question by a study of cycloheximide resistant strains of *Saccharomyces cerevisiae*, which were obtained from normally sensitive strains by gene mutation. Some of these mutants appeared freely permeable to the drug, so that the mechanism of resistance had to be searched for inside the cell. COOPER et al. (1967) found that the incorporation of amino acids into protein was inhibited in a cell free system of the wild strain, but much less in such a system of the resistant strain. They then combined ribosome and supernatant fractions from sensitive and resistant strains in various ways. When the ribosome fraction from the wild strain was incubated with the supernatant of the resistant strain, no resistance to cycloheximide was obtained; when, however, the ribosome fraction of a resistant strain was combined with the supernatant of a wild strain, resistance to cycloheximide did occur. Selective action of cycloheximide in this case may be attributed to the nature of the ribosomes. It is, however, not yet known in what way the ribosomes in the resistant strain differ from those in the sensitive strain.

The significance of differences between ribosomes of sensitive and resistant micro-organisms was indicated by research with streptomycin, an antibiotic with systemic activity against various bacterial and some fungal plant diseases. It is known that also this antibiotic interferes with protein synthesis. In contrast to cycloheximide, it kills bacteria, not fungi. Through mutation bacteria can become resistant to streptomycin, and this resistance appeared to be located in the ribosome, namely in the 30S part. NOMURA (1969) analysed in a brilliant study the various components of the 30S part of the ribosomes in *Escherichia coli*. This part could be broken down to a 16S RNA unit and 19 proteins, and again reconstituted from these components. By reassembling ribosomes from components of resistant and sensitive ribosomes, the factor responsible for resistance could be located. It appeared to be one of the 19 proteins. In this case selectivity of streptomycin was thus due to the protein composition of the ribosome. It seems possible that slight differences in the ribosomes or other parts of the metabolic machinery of organisms, such as mentioned above, may also be responsible for the hitherto unexplained selective action of various of the newly developed systemics.

Selective Systemics for Practical Use

A purposeful search for systemic fungicides is difficult, since we know too little about the metabolic differences in plants and pathogens. Most compounds with systemic action against fungal and bacterial plant diseases, discovered in laboratory and greenhouse screening tests, appeared to be more or less toxic to the host plant. This should not be surprising because of the close relationship between fungi and plants. At present, we may be a little more optimistic, since research into the mechanism of action has revealed some principles upon which a

selective action can be based, and since, albeit by trial and error, some highly selective systemic compounds have been developed, which show much promise for practical use.

A very selective action of a systemic against plant diseases may, however, also be a drawback for its commercial development, because the market for such a compound may be too small. An example is N-phthalimido-diethylphosphonothionate (Dowco 199), which is excellent for the control of rose powdery mildew. It is, however, not available to the grower, since the company concerned does not produce it anymore, as it seems for economical reasons.

Development of Resistance

Development of resistance in fungi to fungicides has not yet caused great difficulties in the control of plant diseases. Since most of our common protectant fungicides are relatively non-selective and react with many enzyme systems, the development of a high degree of resistance can not readily be expected.

The situation might be different for fungicides which act selectively on the metabolism of fungi, as is the case with various systemic fungicides (DEKKER, 1969). Recently, resistance to benomyl and to certain pyrimidine derivatives has been encountered after application of these compounds in the field or in the greenhouse. It would not be very surprising if a parasite would acquire resistance to biochemically selective agents more readily than to general enzyme poisons.

In the Laboratory of Phytopathology we have obtained some experience with acquired resistance to such a selective compound, namely with the fungus *Cladosporium cucumerinum* and the systemic fungicide 6-azauracil. When spores of this fungus were irradiated with UV, a number of strains were obtained which tolerated high concentrations of AzU. As has been mentioned earlier, AzU, in order to be fungicidal, has to be converted in 6-azauridine (AzUR) and 6-azauridine-5'-phosphate (AzUMP). From autoradiographic studies it appeared that part of the resistant strains were not able to convert AzU into AzUR, so that no lethal synthesis occurred (DEKKER, 1968). Other strains, which did convert AzU into AzUR, showed a reduced conversion of AzUR to AzUMP, resulting in increased tolerance to AzU (DEKHUIJZEN and DEKKER, 1970).

The question arises what may be done to counteract the development of resistance to systemic fungicides in pathogenic fungi. We might consider the following possibilities:

1. Mixing of compounds which attack the fungal organism at different sites in its metabolism; this will reduce the chance for development of resistant strains considerably.

2. Changing to a different systemic fungicide after a period of use; after one or more changes the original compound might be used again.

3. Use of a systemic fungicide, which attacks the metabolism of the patho-

gen at more than one site; it must be admitted, however, that it may be hard to combine this property with high selectivity.

4. Use of a systemic non-fungicidal compound, which provides control of diseases in an indirect way.

It is, however, still difficult to predict how the newly developed systemics will perform in this respect in the future. As I mentioned before biological selectivity does not necessarily coincide with biochemical selectivity, and it is to be expected that it is the last type of selectivity, which enhances the risk of the emergence of resistant strains.

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Mechanism of Resistance of *Cladosporium cucumerinum* against 6-Azauracil and 6-Azauridine

By

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The mechanism of resistance of strain R of *Cladosporium cucumerinum* resistant to 6-azauracil (AzU), 6-azauridine (AzUR) and to 6-azauridine-monophosphate (AzUMP) has been studied. Conversion of AzU into AzUR proceeds in both strains equally well. Both the wild strain (N) and the resistant strain (R) convert AzUR into AzUMP. However, the amount of AzUMP detected in the resistant strain was approximately three times lower than in strain N. The poor ability to form AzUMP from AzUR by strain R resulted in a low degree of orotidine monophosphate decarboxylase inhibition, in this way interfering less with the incorporation of orotic acid into RNA. The defect in pyrimidine metabolism at the stage of uridine kinase accounts also for the lower incorporation of AzU into RNA of strain R as compared with strain N. Resistance of strain R to AzUMP depends on the inability of AzUMP to reach in sufficient amounts the enzyme OMP-decarboxylase.

Six years ago at the conference on Host—Parasite Relations in Plant Pathology in Budapest, OORT and DEKKER (1964) discussed the systemic action of 6-azauracil against powdery mildew. 6-azauracil (AzU) is not only active against powdery mildew but also against other fungi such as *Cladosporium cucumerinum*.

DEKKER (1962, 1968, 1969), DEKKER and OORT (1964) studied the mode of action of this compound and found that it interferes with the normal pyrimidine biosynthesis of *C. cucumerinum* (Fig. 1).

6-azauracil is the N_6-analog of uracil and the enzymes uridine phosphorylase and uridine kinase which convert uracil into uridine and subsequently into uridine-5′-phosphate (UMP) also convert AzU into azauridine (AzUR) and into azauridine-5′-phosphate (AzUMP). AzUMP inhibits decarboxylation of OMP into UMP. Apparently, AzU itself is not toxic but has to be converted along the pathways of lethal synthesis to the final toxic compound AzUMP. The fact that AzU itself is a harmless compound becomes also clear from experiments with strains of *C. cucumerinum* resistant against AzU. DEKKER (1968, 1969) obtained a mutant strain after UV irradiation of spores of the wild strain N which was resistant against AzU but not to AzUR and AzUMP. 2-^{14}C-AzU was fed to the spores of this strain and it appeared that the strain was unable to convert AzU into AzUR. In similar experiments it was found that the strain did not convert the normal metabolic uracil into uridine. Apparently, this strain contains an al-

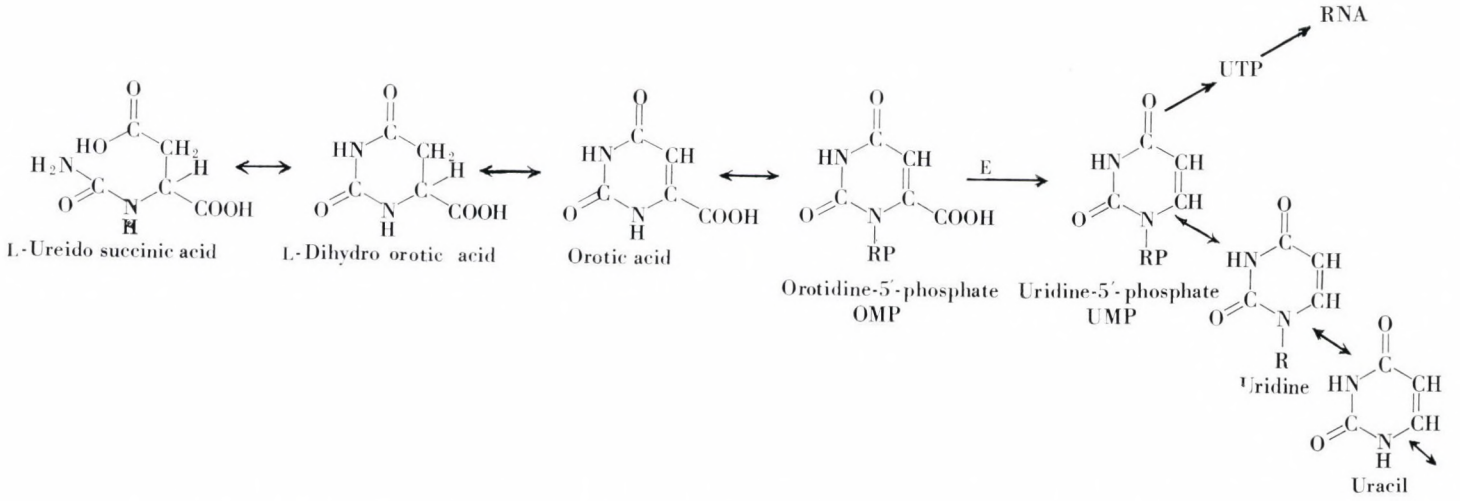

Fig. 1. Part of pyrimidine biosynthesis. AzUMP inhibits the enzyme OMP-decarboxylase (E), which catalyses the conversion of OMP into UMP. UTP = uridinetriphosphate, RNA = ribonucleic acid

tered uridine phosphorylase enzyme which is not able to form sufficient amounts of AzUR.

This report deals with another UV mutant of *C. cucumerinum* (strain R) which is resistant not only to AzU but also to AzUR and AzUMP. To elucidate the mechanism of resistance, the fate of 2-^{14}C-AzU (4 μC) in the wild strain N and strain R was studied. The compound was fed to the spores and six days later RNA was extracted with tris-chloride buffer and sodium lauryl sulfate. RNA and DNA were separated from proteins with phenol and precipitated from the aqueous layer with ethanol. Data showed a low but distinct incorporation of ^{14}C from AzU into RNA of both strains. However, ^{14}C was more than twice as much incorporated into RNA of the wild strain than in that of strain R (621 and 221 dpm/mg RNA).

Table 1

OMP-decarboxylase activity in spores of strain N and R 7 days after application of AzU ($4 \cdot 10^{-4}$M). Mycelia were homogenized in phosphate buffer, pH 6.2. After centrifugation $^{14}CO_2$ evolved has been measured for 15 min after application of 0.05 μC ^{14}COOH-OMP to 5 ml aliquots of the supernatant

Strain	% Growth inhibition	% Reduction of OMP-decarboxylase activity
N	43	92
R	17	30

Chromatography of the aqueous layer showed no difference in the formation of AzUR, but strain R yielded a three times lower amount of AzUMP than strain N (Fig. 2). From this result it appears that strain R and N convert AzU into AzUR equally well, whereas strain R convert AzUR at a much lower rate into AzUMP than strain N. This conclusion was confirmed by feeding ^{3}H-AzUR (10 μC) to the spores for 6 days. Chromatography of mycelial extracts revealed the formation of AzUMP by strain N but not by strain R. In addition, feeding of 2-^{14}C uridine (1 μC) for two hours yielded a three times lower amount of UMP with strain R than with strain N. Most probably a reduced activity of the enzyme uridine kinase accounts for the inability of strain R to synthesize significant amounts of UMP or AzUMP from uridine or azauridine.

From these results it can be expected that the lower AzUMP level, gives rise to a less effective inhibition of the enzyme OMP-decarboxylase in the resistant strain. To investigate this, AzU was fed for 7 days to the spores of both strains. Mycelia were harvested, homogenized and then the substrate carboxy-^{14}C-OMP added to the crude enzyme preparation.

Table 1 shows that OMP-decarboxylase activity of strain N is much stronger reduced than that of strain R. However, the degree of growth inhibition is not directly related to the degree of reduction of OMP-decarboxylase activity.

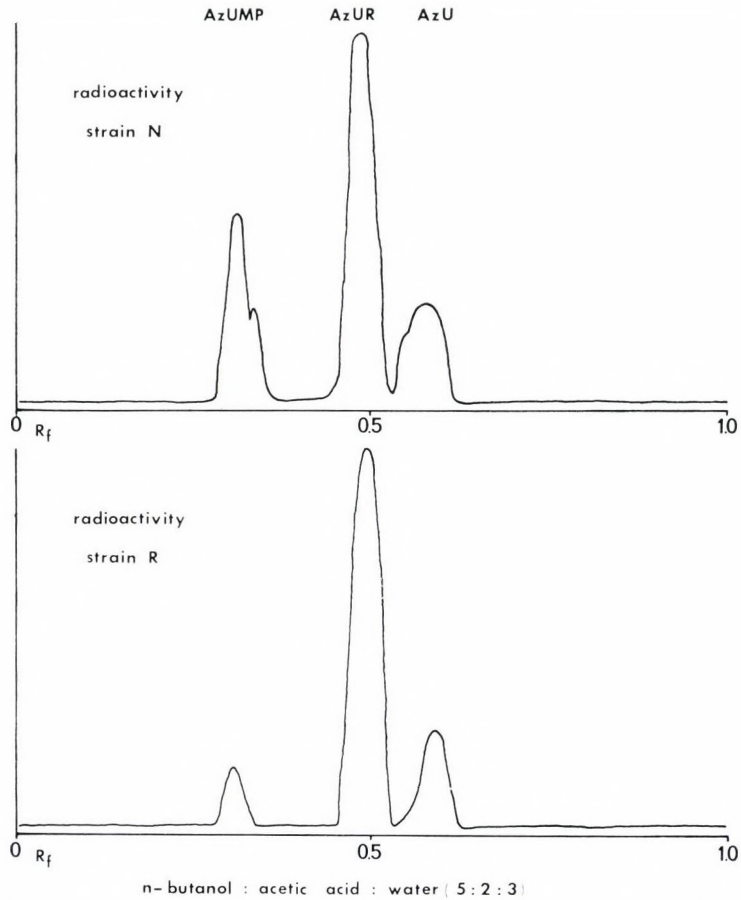

Fig. 2. Conversion of 2-^{14}C-AzU into AzUR and AzUMP by spores of strain N and R

Similar results were obtained in experiments in which the incorporation of 2-^{14}C-orotic acid into RNA in presence of AzU was studied. As compared to the resistant strain, incorporation into RNA is more reduced in strain N. It is therefore apparent that the resistance of strain R to AzU depends mainly on the failure to form significant amounts of AzUMP from AzUR. This results in a concurrent low inhibition of OMP-decarboxylase and a low degree of incorporation of AzU into RNA. However, if this holds true, one would expect that application of AzUMP would strongly inhibit growth of strain R. Strain R, however, is also resistant to AzUMP. To clarify this situation AzUMP was fed for 18 hours to spores of both strains. Subsequently the spores were washed and homogenized. Table 2 shows decarboxylase activity of the homogenate.

In contrast to the enzyme activity of strain N, the activity of strain R is not reduced at all. A close correlation exists between resistance of strain R and the

Table 2

OMP-decarboxylase activity in spores of strain N and R 18 hours after application of AzUMP ($4 \cdot 10^{-4}$M). $^{14}CO_2$ evolved has been measured for 15 min after application of 0.05 μC ^{14}COOH-OMP.

Strain	% Inhibition of growth of germ tube	% Reduction of OMP-decarboxylase activity
N	45	59
R	0	0

non-reduced activity of the enzyme OMP-decarboxylase. From these experiments it seems highly probable that differences in reaching the enzyme OMP-decarboxylase inside the cells play a decisive role in the differences in sensitivity of strain N and R to AzUMP. This may be due to a decreased permeability of the resistant cells to AzUMP or this compound is rapidly broken down before reaching the site of action. PASTERNAK et al. (1961) assume that resistance of leukemia cells to AzUMP is due to a rapid cleavage of the 5′-phosphate from the compound.

Summarizing, it is clear that (1) resistance to AzU and AzUR is due to a defect at the stage of uridine kinase. (2) Most probably Azu is incorporated into RNA. It seems highly probable that growth inhibition of the wild strain N is caused by a dual action of two different mechanisms. The first mechanism leads to inhibition of OMP-decarboxylase activity or to a repression of enzyme synthesis, in this way interfering with the biosynthesis of RNA and the second one to an incorporation of AzU into RNA. (3) Resistance to AzUMP may depend on the inability of this compound to reach in sufficient amounts the enzyme OMP-decarboxylase. A full account of the results was published recently by DEKHUIJZEN and DEKKER (1970).

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Indirect Action of 6-Azauracil against *Pythium debaryanum* in the Rhizosphere of Cucumber Seedlings

By

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When cucumber seeds were soaked in a 1 ppm solution of 6-azauracil (AzU) for 10 hours, the seedlings grown from these seeds appeared to be protected against damping off, caused by *Pythium debaryanum*. Since this fungus is rather insensitive to AzU in vitro, it has to be assumed that the control of damping off was obtained in an indirect way. Moreover, little or no protection was obtained after treatment of the seeds in a 10 or a 100 ppm solution of AzU. When the experiments were done under sterile conditions no effect of AzU against the disease was observed. This indicates that the action of AzU might take place via the rhizosphere microflora. After treatment of the seeds in a 1 ppm solution of AzU, a substantial increase of the rhizosphere bacteria was obtained. Antagonistic activity of these bacteria towards *P. debaryanum* was higher than that of rhizosphere bacteria of control plants. It is suggested that seed treatment in low concentrations of AzU might change the quality or quantity of the root exudates in such a way that certain bacteria, especially those which use nitrogen in the form of amino acids, are favoured. In preliminary experiments the quantity of amino acids in the root exudates appeared 30–40% higher than in control plants.

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Some Observations on Activity and Metabolism of a New Systemic Fungicide, N,N'-bis-(1-formamido-2,2,2-trichloroethyl)-piperazine (CELA W 524)

By

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A new systemic fungicide, CELA W 524, has been examined with respect to *in vitro* fungitoxicity and antifungal and systemic activity as well as metabolic conversion in plants. The compound exerts a moderate fungitoxic action towards non-obligate parasites and saprophytes when grown on Czapek-Dox-malt agar or in liquid medium; much more pronounced is its inhibitory effect on spore germination *(Aspergillus niger, Cladosporium cucumerinum)* and also, although to a lesser extent, on germ tube elongation. It protects barley and cucumber plants against mildew, when administered via the roots or when sprayed, and provides complete protection of cucumber plants against cucumber scab *(C. cucumerinum)*. On heat sterilization the compound is rapidly broken down to at least two non-fungitoxic compounds. In plants too, it is metabolized to at least two non-fungitoxic compounds. The identity of the non-enzymatic and metabolic breakdown products of W 524 is being investigated.

In 1967, Dr. W. OST of C. H. Boehringer Sohn, Ingelheim am Rhein, Germany, synthesized a new systemic fungicide, N,N'-bis-(1-formamido-2,2,2-trichloroethyl)-piperazine, coded as CELA W 524 (Fig. 1), which was found to be particularly active against rusts, mildews and apple scab (SCHICKE and VEEN, 1969).

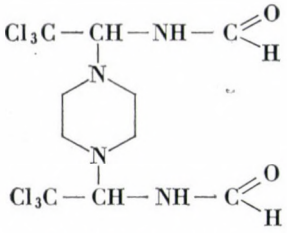

Fig. 1. Structural formula of N,N'-bis-(1-formamido-2,2,2-trichloroethyl)-piperazine (W 524)

Further results on in vitro fungitoxicity and activity of W 524 against plant diseases have been published since (FUCHS et al., 1971). They show, that against most fungi tested (for instance, *Alternaria tenuis, Ascochyta pisi, Botrytis cinerea, Colletotrichum lindemuthianum, Colletotrichum orbiculare, Fusarium oxysporum* f.

pisi, *Glomerella cingulata*, *Stemphylium radicinum*) W 524 displayed only a moderate fungitoxic activity in vitro. On the other hand, it strongly inhibited spore germination and germ tube elongation of *Aspergillus niger* and *Cladosporium cucumerinum*. In addition, a relatively marked inhibition of growth of these two fungi could be observed, when grown on Czapek Dox agar. A thin-layer chromatographic bioassay according to HOMANS and FUCHS (1970) revealed a distinct inhibition of fungal growth by 5–50 μg amounts of W 524 in the case of *Botrytis cinerea*, *Cladosporium cucumerinum*, *Fusarium culmorum* and *Penicillium expansum*. Thus, although W 524 is certainly not a broad spectrum fungicide, it obviously displays a distinct inhibitory effect in vitro towards a number of non-pathogenic and pathogenic fungi.

At concentrations above 25–50 mg a.i./l one of the two available formulations of W 524, which differed from the other in the emulsifier present, caused appreciable phytotoxicity in several plant species, especially upon repeated spraying or when applied via the roots. The other formulation, however, was virtually non-phytotoxic, only concentrations above 50–100 mg a.i./l causing very mild symptoms in some renowned sensitive plant species, like cucumber and tobacco.

Especially upon spraying before (artificial) inoculation the non-phytotoxic formulation provided excellent control of some powdery mildew diseases (barley, cucumber and pea powdery mildew, caused by *Erysiphe graminis* f. sp. *hordei*, *Sphaerotheca fuliginea* and *Erysiphe pisi*, respectively), but it also caused considerable to complete protection against bean rust *(Uromyces appendiculatus)*, cucumber scab *(Cladosporium cucumerinum)*, and tomato leaf mould *(Cladosporium fulvum)* at concentrations of 5 or 25 mg/l. Further, after application via the roots it proved to be systemically active protecting barley plants against powdery mildew at concentrations as low as 1.5 mg a.i./l and cucumber plants against both powdery mildew and scab at concentrations of about 25 mg a.i./l.

W 524 proved to be unstable upon heat sterilization, at the same time losing its fungitoxicity completely as could be revealed by the thin-layer chromatographic bioassay mentioned before (Fig. 2). When aqueous solutions of W 524, to which small amounts of ^{3}H-labelled W 524 had been added, were chromatographed with ether as the solvent system the spot containing the greater part of the radioactivity (cca 75%) coincided with the fungitoxic spot (Fig. 3); less activity (cca 20%) was retained in a non-moving substance which, in addition, was not fungitoxic. Upon heat sterilization almost all radioactivity (98–99%) was retained in a non-moving, non-fungitoxic spot, which, moreover, turned bright yellow upon spraying with $KMnO_4$. Piperazine-hydrate, used as a reference, behaved in exactly the same way. Therefore, it was tempting to test the hypothesis that W 524 upon heat sterilization is decomposed giving rise to piperazine as the only ^{3}H-labelled compound. The results of experiments, in which *n*-butanol/acetic acid/water 4 : 1 : 1 was used as the solvent system, obviously disproved this idea: although with this solvent in non-heated solutions again two radioactive spots were observed, with R_f-values 0.95 and 0.55, the first one being fungitoxic, upon heat sterili-

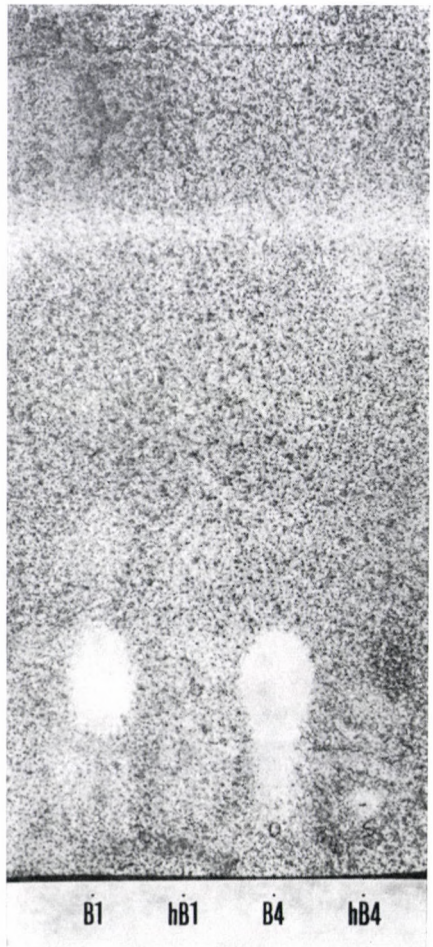

Fig. 2. Thin-layer chromatogram (DC-Alufolie Kieselgel F 254, Merck), showing instability of W 524 upon heat sterilization. B1, B4: different formulations of W 524; hB1, hB4: the same formulations after heating at 120°C for 20 min. The quantity spotted represented in each case 10 μg active ingredient. Solvent: ether; chromatogram sprayed with a conidial suspension of *Cladosporium cucumerinum*

zation a series of non-fungitoxic decomposition products were formed. Two of these, with R_f-values 0.85 and 0.45, retained cca 50 and 35% of the radioactivity, respectively. Three other, more slowly moving and only weakly radioactive spots, with R_f-values 0.34, 0.17, and 0.03, reacted with $KMnO_4$ giving bright yellow spots. As is shown in Fig. 3 the latter two co-chromatographed with N-formyl-piperazine and piperazine, respectively. The third one behaved differently from N,N'-di-

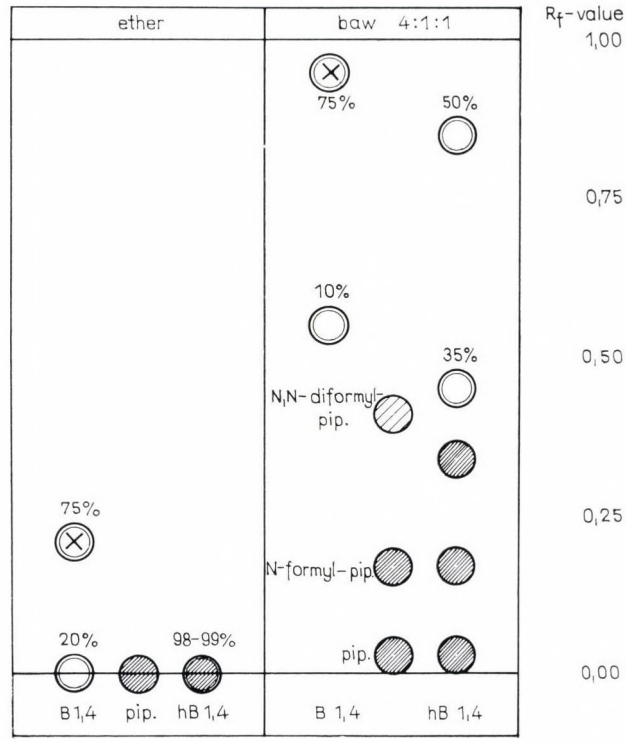

Fig. 3. Diagrammatic chromatogram, showing conversion of W 524 to non-fungitoxic substances upon heat sterilization. Bold-faced circles (◎): radioactive spots; shaded circles (◍): spots reacting with $KMnO_4$; circles with cross (⊗): spots fungitoxic to *Cladosporium cucumerinum*; B1, B4, hB1, hB4: for explanation see Fig. 2.; pip., N-formyl-pip., N,N'-diformyl-pip.: piperazine-hydrate, N-formyl-piperazine, N,N'-diformyl-piperazine. Solvents: ether and *n*-butanol : acetic acid : water 4 : 1 : 1

formyl-piperazine, another compound used as a reference, in two respects: it showed a somewhat lower R_f-value, and gave a much brighter yellow spot. It might be too speculative to suggest that it is also a mono-N-substituted piperazine derivative. Prolonged heat sterilization of W 524 proved to give rise to higher proportions of radioactivity in the spots with lower R_f-values, especially in that co-chromatographing with piperazine. Detailed reports will be published elsewhere.

After uptake of W 524 by a variety of plants, viz. barley, wheat, Chinese cabbage, cucumber, tobacco and *Vigna* bean, it was the only fungitoxic compound present, as was shown by a thin-layer chromatographic bioassay using *Cladosporium cucumerinum* as the test organism (Fig. 4).

Metabolic conversion of W 524 in relation to time has been studied in a variety of plants and host-parasite combinations, viz. barley and bean, and healthy

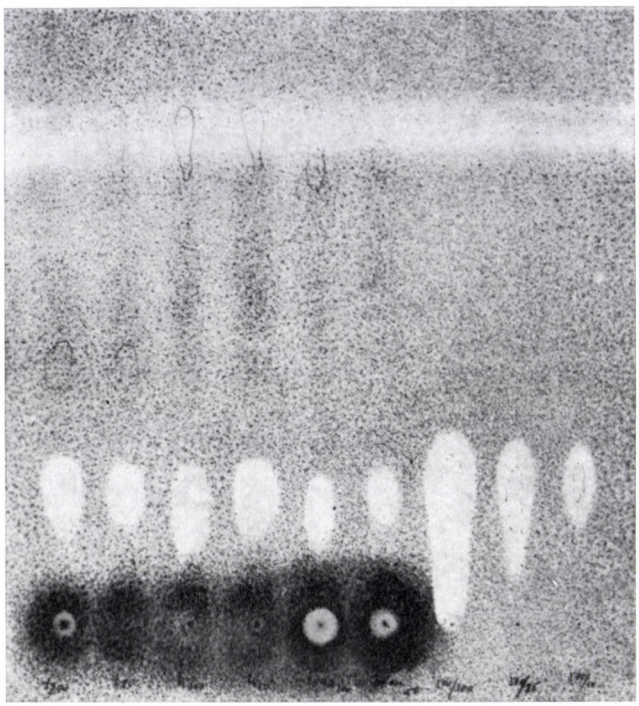

Fig. 4. Thin-layer chromatogram, showing unchanged presence of W 524 in plants after uptake via the roots. From left to right: tobacco (2×), Chinese cabbage (2×), bean (2×), and reference W 524 (3×; 50, 12.5 and 5 μg, respectively). Solvent: ether; chromatogram sprayed with a conidial suspension of *Cladosporium cucumerinum*

and *Cladosporium cucumerinum*-infected cucumber plants, and healthy and *Ascochyta pisi*-infected pea plants. While standing in small vials, the plants were allowed to take up via their root system the equivalent of 10 – 500 μl ^{3}H-labelled W 524, representing 1 – 50 μg a.i., dissolved in 200 – 500 μl of aqueous solutions of cold W 524, which contained 50 mg a.i./l. After this liquid and at least two more 200 – 500 μl aliquots of non-labelled W 524 had been taken up completely, the vials were filled with 10 ml of an aqueous solution of cold W 524. After 24 hours, these solutions were replaced by 10 ml Hoagland solution; from that time onwards, plants were sacrificed periodically and examined for radioactivity. To this end, the plants were squeezed out producing the so-called sap; the remainder was extracted afterwards with 96% ethanol. Sap and ethanolic fraction were chromatographed separately and in duplicate, using ether and/or *n*-butanol/acetic acid/water 4 : 1 : 1 as the solvent systems. Whereas one chromatogram of each type was bioassayed, the other served to locate radioactive spots. Only a few results, which are representative for all experiments, will be discussed here. Details will

Table 1

Percentage of radioactivity at different R_f-values of ether developed thin-layer chromatograms of sap, resp. ethanolic extracts of barley plants (cv. Cambrinus), which were allowed to metabolize ^{3}H-labelled W 524 for 1, 8, and 16 days, respectively; B1, B4: two different formulations of W 524

Days	B1			B4		
	1	8	15	1	8	15
Sap						
R_f 0.00—0.10	50.7	69.3	78.1	51.6	71.3	69.8
R_f 0.10—0.20	2.5	2.0	3.9	2.7	1.6	3.9
R_f 0.20—0.30*	46.7	28.7	18.0	45.7	27.0	26.3
Ethanolic extract						
R_f 0.00—0.10	16.1	27.9	37.7	13.3	24.0	36.3
R_f 0.10—0.20	1.0	1.3	1.4	1.1	0.9	2.1
R_f 0.20—0.30*	82.9	70.5	60.8	85.6	75.1	61.6

* zone of fungitoxic activity

Table 2

Percentage of radioactivity at different R_f-values of *n*-butanol/acetic acid/water 4 : 1 : 1 developed thin-layer chromatograms of sap, resp. ethanolic extracts of healthy and *Ascochyta pisi*-infected pea plants (cv. Mansholt), which were allowed to metabolize ^{3}H-labelled W 524 (formulation B4) for 1, 5, and 8 days, respectively

Days	Healthy plants			*A. pisi*-infected plants		
	1	5	8	1	5	8
Sap						
R_f 0.50—0.60	11.2	35.8	41.9	11.2	34.6	44.4
R_f 0.80—0.90	16.5	8.2	13.3	16.5	5.3	7.2
R_f 0.90—1.00*	53.9	26.9	14.4	53.9	26.5	15.6
Ethanolic extract						
R_f 0.50—0.60	2.7	6.3	14.5	2.7	11.2	15.3
R_f 0.80—0.90	9.4	21.2	10.6	9.4	21.9	21.9
R_f 0.90—1.00*	84.1	63.5	65.4	84.1	57.2	48.9

* zone of fungitoxic activity

be published elsewhere. Table 1 shows the percentages of radioactivity in the two main radioactive spots in ether-developed chromatograms of barley plants in relation to time. As can be seen, a compound coinciding and most probably identical with W 524 (R_f-value 0.20—0.30) was gradually converted to (a) non-moving compound(s) (R_f-value 0.00—0.10). Evaluation of the data on radioactivity in sap, resp. ethanolic fraction, leads to the conclusion, that the latter compound

is more water-soluble than the original compound W 524, which is, indeed, almost insoluble in water. Again, as in the case of thermal decomposition of W 524, it was tempting to suggest that the conversion product, which was also found in all other plants and host-parasite combinations examined, is identical with piperazine. But again, chromatography with *n*-butanol/acetic acid/water as the solvent system proved this assumption to be incorrect; almost all radioactivity was retained in exactly the same two spots as originally present in non-heated solutions of W 524. However, as is shown in Table 2 for healthy and *Ascochyta pisi*-infected pea

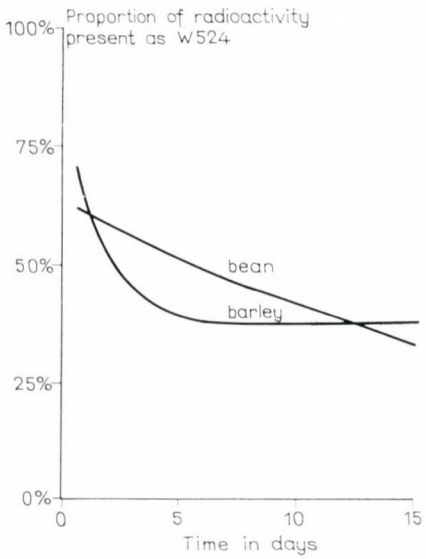

Fig. 5. Proportion of radioactivity present as W 524 in relation to time in barley and bean plants, which were allowed to take up via their root system 10 μl ^{3}H-labelled W 524 (spec. act. 34.23 μC/ml)

plants, a gradual change with time in the distribution of radioactivity over the two spots obviously indicates a gradual conversion of the original fungitoxic compound W 524 (R_f-value 0.90 – 1.00) into another, in fact non-fungitoxic compound (R_f-value 0.50 – 0.60). Due to the technique used, the data do not permit a reliable conclusion on whether this conversion took place via a compound with R_f-value 0.80 – 0.90 or whether a (non-enzymatic?) breakdown of W 524 to the latter compound proceeded concomitantly; at any rate, upon degradation of W 524 significant 'amounts' of radioactivity appeared temporarily in this zone with R_f-value 0.80 – 0.90. From the available data, the proportion of W 524 among the radioactive conversion products in relation to time can be calculated; Fig. 5 shows, that in healthy barley and bean plants this proportion decreases gradually from

cca 65 to 35%, within 16 days. In other species, like cucumber, breakdown seemed to proceed more rapidly. Infection affected the rate of breakdown only to a small extent; in fact, W 524 was degraded only a little faster in infected plants.

About the nature of this degradation only speculations can be made, so far. The high reactivity of the trichloro-substituted methyl group renders breakdown of the molecule with production of chloroform quite likely; this might be the reason of the loss of fungitoxicity of W 524. As final products, N,N'-diformyl-, N-formyl-, and unsubstituted piperazine could be considered, although neither thermal decomposition – at least when short periods of heat sterilization were employed – nor enzymatic degradation gave rise to a high proportion of radioactivity in these compounds. N,N'-diformyl-piperazine might constitute an exception; there is some circumstantial evidence, that it might be identical with the radioactive spot in the zone with R_f-value 0.40–0.50 (*n*-butanol/acetic acid/water 4 : 1 : 1). A closer examination into the identity of these compounds, currently in progress, might reveal whether these speculations are justified.

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Metabolism and Mode of Action of Dimethirimol and Ethirimol

By

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Ethirimol and dimethirimol are the most active of a group of hydroxypyrimidines showing specific control of powdery mildews. Both compounds are readily metabolized in plants following uptake by the roots. Loss of one N-methyl group from dimethirimol occurs very quickly in cucumber plants giving the N-desmethyl derivative which is a very active fungicide. The second N-methyl group is lost more slowly giving the $—NH_2$ compound, which is almost inactive as a fungicide; the same compound is formed as a metabolite of ethirimol (by loss of the ethyl group) in barley plants. Both ethirimol and dimethirimol are also converted into a complex mixture of water-soluble metabolites, some of which are fungicidally active and believed to be conjugates of glucosides and/or phosphates. Thus, mild hydrolysis of the water-soluble metabolite fraction from ethirimol gives back ethirimol, ethirimol glucoside, des-ethyl ethirimol and a minor product in which hydroxylation of the butyl group has occurred. Both compounds are metabolized and excreted following oral administration to rats. A comparative metabolism in animals and plants is discussed. Ethirimol and dimethirimol are fungicidal within the host plant leaves at concentrations below 10^{-7} *M* and are consequently believed to be non-competitive enzyme inhibitors. They possibly act as antagonists to pyridoxal, which is involved through C-1 metabolism in the biosynthesis of purines, thymidine and amino acids, as well as a variety of other classes of enzyme reaction.

Dimethirimol (PP 675) and ethirimol (PP 149) are two closely related compounds selected for development from a group of pyrimidine fungicides which give specific control of powdery mildews on a range of plants. The compounds differ only in the nature of the amino group in the 2-position of the pyrimidine ring (Fig. 1). Both show systemic activity in herbaceous plants, but not in woody tissue. Ethirimol is intrinsically the more active chemical and is superior to dimethirimol for the control of mildews of cereals and grasses. It has been introduced under the trade name 'Milstem' for the control of powdery mildew *(Erysiphe graminis hordei)* of barley.

Because of its more suitable physical characteristics, particularly its higher water solubility, dimethirimol was selected and developed (under the trade name 'Milcurb') for powdery mildew control of cucumbers and other cucurbit crops. In this case the chemical is applied as a solution to the soil containing the root zone. When applied in this way a single soil drench gives complete protection both

Fig. 1

Fig. 2

to pre-existing and to newly formed leaves for at least eight weeks. Fig. 2 shows the difference between treated and untreated glasshouse cucumber plants eight weeks after applying a solution of dimethirimol to the soil. Both compounds are adsorbed to soil and are not readily leached. Best results are consequently achieved when they are applied in the root zone. Local application of ethirimol, as for example by seed dressings, gives rise to high local concentrations. Persistence of this zone of high concentration is probably responsible for the persistence of mildew control in plants.

Translocation

Upward movement of these fungicides occurs in the xylem, but they are not translocated downwards in the phloem. Consequently, although they are active as foliar sprays, only leaves present at the time of spraying will be protected. When droplets of the chemicals are applied to leaves, a proportion enters and moves upwards towards the tip and margin of the leaf and also from one side of the leaf to the other. A droplet applied at the base of a leaf will protect the whole of that leaf and control mildew on it. Thus, if properly timed and applied, spray applications can give very good disease control. However, soil applications (e.g. as a seed dressing for 'Milstem') enable the chemical to move continuously through the whole plant, giving protection whilst the chemical persists.

Fig. 3 shows autoradiographs of barley plants at different stages after treatment of the roots with ^{14}C-ethirimol ('Milstem'), and Fig. 4 shows a similar autoradiograph of cucumber plants treated with radioactive dimethirimol. There is no significant redistribution of radioactivity from original leaves to new leaves after the root source of radioactivity is removed.

^{14}C-labelled ethirimol and dimethirimol have been synthesized with the radioactive carbon in the 2-position of the pyrimidine ring. Figure 5. shows the synthesis of ^{14}C-ethirimol. Radioactive dimethirimol is prepared by using dimethylamine in the last stage.

A full study of the metabolism of these compounds in plants has been carried out by Dr. B. D. CAVELL and Dr. R. J. HEMINGWAY at Jealott's Hill Research Station and in animals by Dr. J. W. DANIEL at Industrial Hygiene Research Laboratories. I would like to describe the animal work with dimethirimol first because it is most complete.

In animals: When fed to rats ^{14}C-dimethirimol undergoes extensive metabolism. The greater proportion (80%) of the radioactivity is excreted within 48 hours in the urine and is associated with five metabolites, each of which has been isolated and characterized. There was no evidence for the excretion of any dimethirimol and the major routes of metabolism involve both the dimethylamino- and *n*-butyl substituents. The dimethylamino group is progressively dealkylated and the *n*-butyl substituent undergoes oxidation at the carbon adjacent to the terminal carbon of the chain.

Fig. 6 shows the structures of these metabolites which were isolated from the urine by ion-exchange chromatography and separated by the counter-current distribution between phosphate buffer and ethyl acetate.

The sixth metabolite was isolated from the bile using ionexchange chromatography and was shown to be the O-glucuronide of dimethirimol. It was isolated as a crystalline compound and its structure confirmed by hydrolysis to dimethirimol and glucuronic acid. The infra-red spectrum of the conjugate provided evidence that it was an O- and not an N-glucuronide.

Metabolism of ^{14}C-ethirimol in the rat follows an identical pattern – the

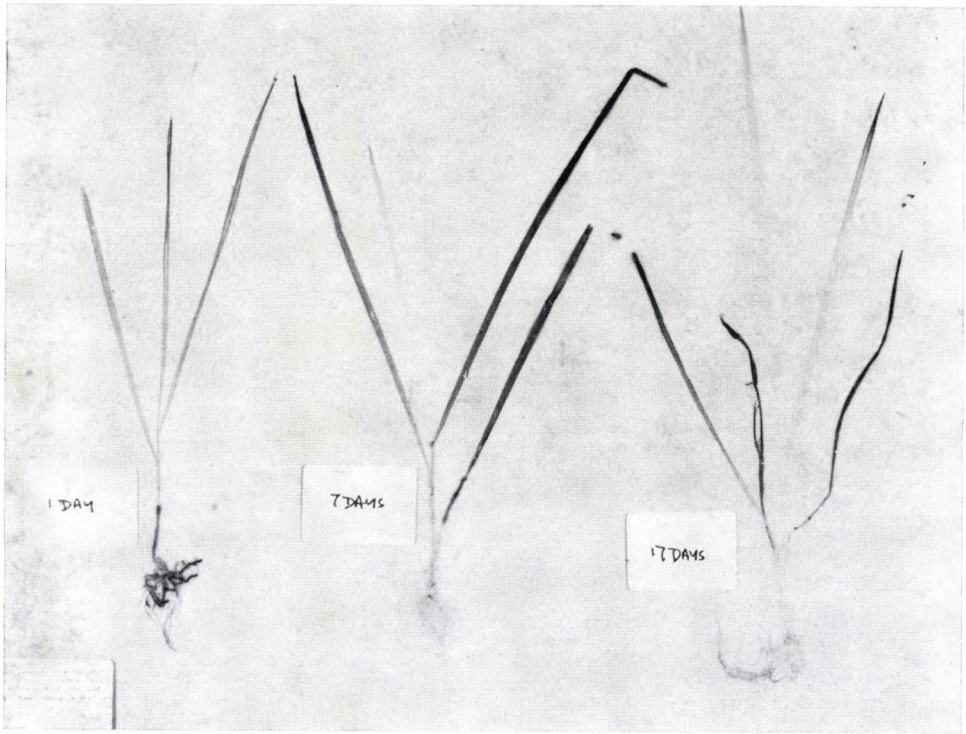


Fig. 3

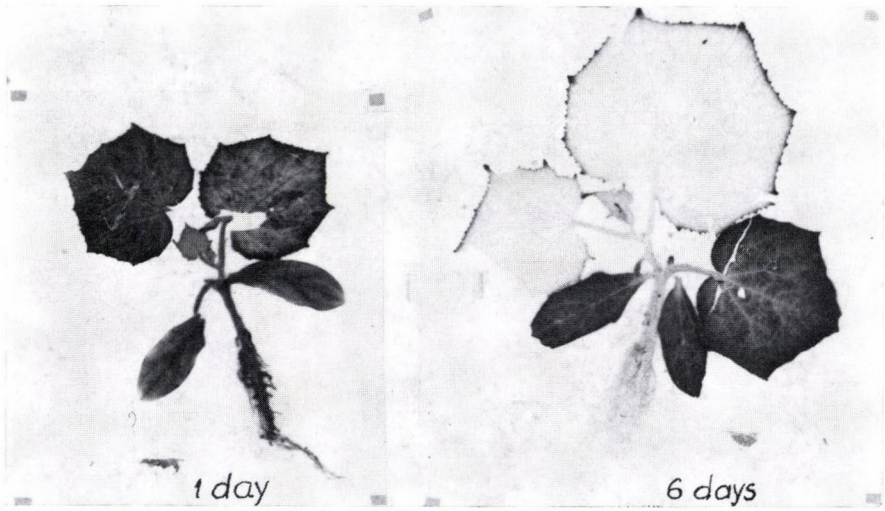


Fig. 4. Translocation of dimethirimol in cucumber seedlings. 1. Root fed 8 hrs; 2. Grown on in water

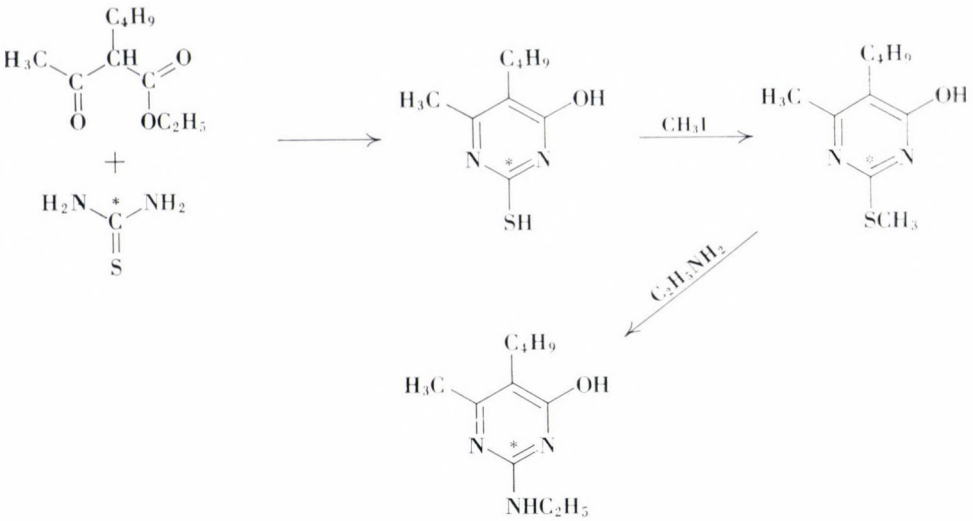

C_4H_9
H_3C
CH
C
O
OC_2H_5
H_2N
NH_2
S
OH
SH
CH_3I
SCH_3
$C_2H_5NH_2$
NHC_2H_5

Fig. 5

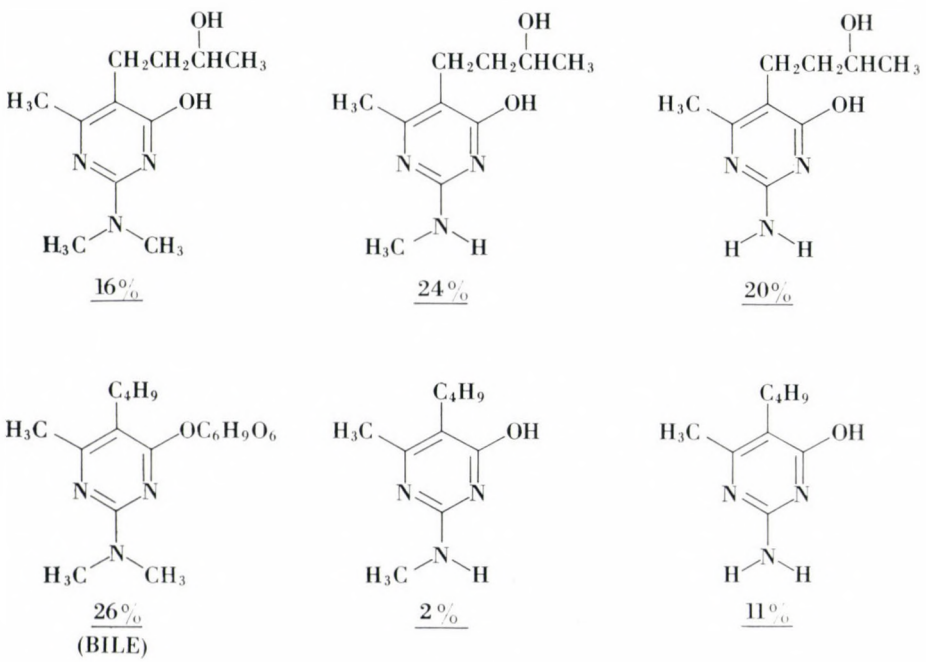

OH
$CH_2CH_2CHCH_3$
H_3C
CH_3
16%
24%
20%
C_4H_9
$OC_6H_9O_6$
26%
(BILE)
2%
11%

Fig. 6

three metabolic routes being N-dealkylation, hydroxylation of the butyl group and formation of ethirimol-O-glucuronide. Almost all the radioactivity is excreted within 48 hours.

Metabolism in plants: ^{14}C-ethirimol is fairly rapidly degraded when fed to barley plants via the roots and has a half life in the plant of approximately 3—4 days. The pattern of metabolism is similar to that observed in animals. The radioactivity extracted from the plant can be partitioned between ether and water, the water-soluble metabolites increasing with time, as shown in Fig. 7. The ether was shown to contain unchanged ethirimol together with the N-dealkylated derivative, whilst the aqueous fraction appeared to contain a complete mixture of products (Fig. 8). After mild hydrolysis of the aqueous fraction, 60% of the radioactivity could be extracted back into ether (or ethyl acetate), and the mixture separated by thin-layer chromatography. Four of the products have been identified by two dimensional co-chromatography with authentic samples. These are

Ethirimol
Des-ethylethirimol (i.e. the N-dealkylated product)
Ethirimol glucoside
Hydroxylated butyl ethirimol (minor metabolite).

A further one major and two minor metabolites in this extract remain unidentified.

Thus it appears that ethirimol and its dealkylated product form conjugates (possibly phosphates) which easily hydrolyse to the parent compound. The appearance of ethirimol glucoside in the hydrolysate suggests the original presence of ethirimol glucopyranoside phosphate. The aqueous fraction is also fungicidal which explains why the biological half life of ethirimol is greater than the chemical half life in plants.

Thus the metabolism of ethirimol in plants follows a similar pattern to that observed in animals and the three routes of metabolism, viz. N-dealkylation, hydroxylation of the butyl group and conjugate formation have all been observed. In the case of plants, however, the hydroxylation reaction appears to be a relatively minor pathway, in contrast to conjugate formation. In plants the conjugates formed are glucosides and probably phosphates, whilst in animals ethirimol glucuronide was the only conjugate identified.

A detailed study has also been made of the residues of ethirimol and its metabolites in barley and wheat plants and grain following application of ^{14}C-labelled ethirimol as a seed dressing in the field. Radioactive fungicide was applied to barley seeds, and the resulting plants were analysed for content of radioactivity at intervals. After one month, the level of radioactivity in the plant represented 2.2 ppm of ethirimol. At harvest, the total residue levels in the plants were very low. In the grain a total of 0.05 ppm of metabolites derived from ethirimol was present, and of this less than 0.002 ppm was ethirimol. In the straw total metabolites were 0.12 ppm and of this less than 0.006 ppm was ethirimol. An autoradiograph of a

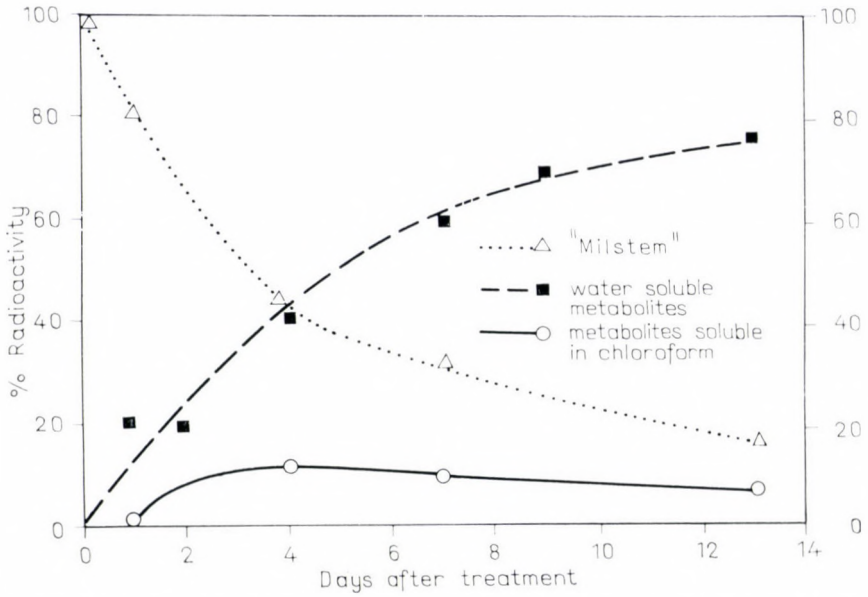


Fig. 7. Radioactivity in extract of barley fed with "Milstem"-C^{14} to the root

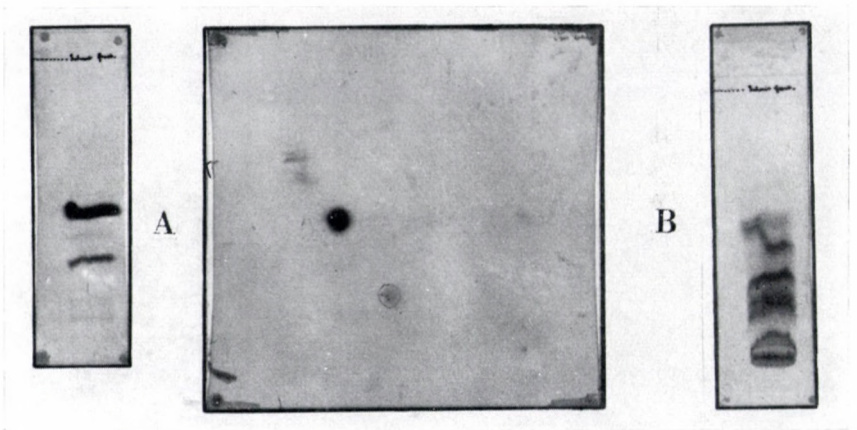
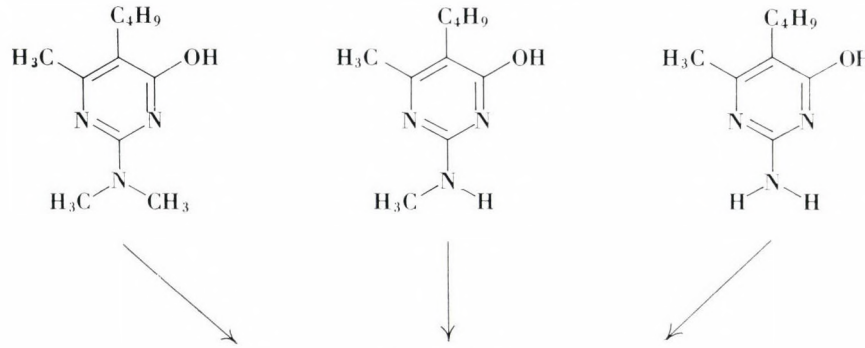


Fig. 8. Barley plants ^{14}C-ethirimol root fed. Analysed after 7 days. A Ether-soluble metabolites. B Water-soluble metabolites

plant just after grain formation shows fairly uniform distribution of radioactivity on the leaves, but very little in the ear.

The metabolism of dimethirimol in cucumber seedlings has also been extensively studied. Dimethirimol-2-C^{14}, fed to young cucumber plants grown in



Conjugates
Fig. 9

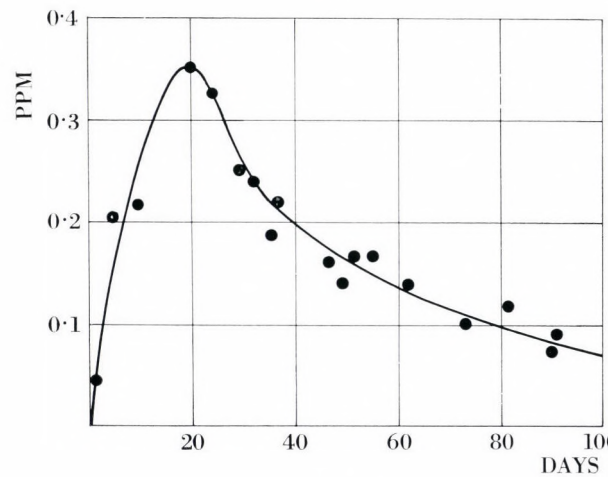


Fig. 10

culture solution, is rapidly degraded with a half life of about 24 hours. N-demethylation occurs very quickly to give the fungicidally active N-monomethyl derivative, an analogue of ethirimol (Fig. 9). Loss of the second N-methyl group occurs more slowly to give the relatively inactive 2-amino derivative, also formed from ethirimol in barley plants. As with ethirimol a complex mixture of water-soluble conjugates are also formed, which can be partially converted to these parent pyrimidines, by mild hydrolysis.

Experiments have also been set up to determine residue levels of dimethirimol and its metabolites in cucumber fruit following normal greenhouse treatments. In a typical experiment a cucumber plant was grown to maturity in a large box of compost (100 kg). When mature fruits were present the soil around the stem was treated at day O with 0.25g radioactive dimethirimol formulated as the hydrochloride salt. Fruits were harvested up to 90 days after treatment and analysed

immediately after collection for dimethirimol and total radioactivity. As can be seen from Fig. 10, total radioactivity present reached a maximum of 0.35 ppm approximately 20 days after treatment, and 0.1 ppm of this could be accounted for as dimethirimol.

Extensive residue analyses of fruit grown under commercial conditions has confirmed that residue levels of dimethirimol are less than 0.2 ppm.

Mode of Action

Both ethirimol and dimethirimol have been shown to be fungicidally active within plant leaves at concentrations of the order of $10^{-7}M$ or less. It is thus assumed that they are acting as non-competitive enzyme inhibitors. A biochemical investigation of an obligate parasite presents unusual difficulties. When using the intact organism it must either be taking metabolites from the host or be in the process of dying. Similarly, if compounds are to be supplied to a healthy mildew growth, then they must be supplied via the host plant.

Research into the mode of action of these systemic fungicides has been carried out at Jealott's Hill by Dr. K. J. BENT and Dr. M. J. SAMPSON, but because of the difficulties outlined, no firm conclusions as to the precise action of these fungicides can yet be made.

To try to locate the site of inhibition of fungal metabolism, a number of compounds were fed to the host plant in an attempt to reverse the fungicidal activity on cucumber powdery mildew. Following uptake of ethirimol reversing agents were supplied at concentrations of 10^{-4} to $10^{-6}M$ and the plants infected and maintained in a spore filled atmosphere. Good reversals were obtained with purines, folic acid and to a much lesser extent with thymine. Orotic acid and cytosine gave very much poorer reversals.

These results suggested an inhibition of tetrahydrofolate (THFA) directed C-1 metabolism. No inhibition of either dihydrofolate reductase or N^5N^{10}methylene tetrahydrofolate dehydrogenase was obtained using an enzyme preparation from cucumber powdery mildew. In addition, the fungicides were found not to inhibit the incorporation of labelled formate into purines or into formyl glycinamide ribotide in a cell-free system. Thus the ability of purines to reverse the fungicidal effect contrasted with the absence of any inhibition in some of the enzyme systems required for their biosynthesis.

Some of the reactions in which C-1 units may be donated to THFA are dependant upon the co-enzyme pyridoxal (vitamin B6). The blockage in metabolism may be related to pyridoxal catalyzed reactions since amino levulinic acid, serine, glycine and pyridoxal all reverse the fungicidal action of ethirimol. The pyridoxal dependant enzymes serine hydroxymethylase and the decarboxylases of leucine and glutamic acid have been shown to be present in cucumber powdery mildew, but no inhibition of these enzyme systems could be obtained with ethirimol.

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Respiratory and Photosynthetic Rates in Apple Leaves Treated with the Systemic Fungicide Benomyl

By

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Benomyl sprayed on apple leaves decreases apparent photosynthesis and inhibits by 50 per cent the intensity of Hill reaction in the chloroplasts. This systemic fungicide does not influence the rate of respiration nor changes the activity of isocitric dehydrogenase.

In the present paper we report on the results of experiments on the metabolic effects of benomyl (methyl-1-(butylcarbamoyl)-2-benzimidazole carbamate, E. I. du Pont's "Benlate") on the host plant (apple leaves). The aim was to study the physiological effects of this systemic fungicide on sprayed apple leaves as exemplified by 1) The intensity of apparent photosynthesis, 2) the effect on the Hill reaction in isolated chloroplasts, 3) the respiratory rate and 4) the activity of isocitric dehydrogenase, measured in a model system.

Materials and Methods

Apple plants were grown under normal greenhouse conditions. They were sprayed with benomyl at the rate of 0.05 per cent, every ten days.

The intensity of apparent photosynthesis was measured by the method of WILSON et al. (1969). The experiments were conducted with a Warburg apparatus with a water-filtered illumination from above giving a maximum light intensity of 10,000 lux in the spectral region of 400—722 μm. A 0.2 *M* carbonate buffer was used as a constant CO_2 source in the flasks of the Warburg apparatus. Rates of apparent photosynthesis of four leaf discs (1 cm^2 surface) placed in a Warburg flask were measured by means of attached manometers. Manometric technique outlined by UMBREIT et al. (1959) was applied in these experiments.

The Hill reaction was measured by the method of JAGENDORF and AVRON (1958). The procedure for the isolation of chloroplasts was as follows:

15 g leaf tissue was homogenized in a cold mortar in 25 ml medium containing: 0.4 *M* sucrose, 0.05 *M* Tris buffer, pH 7.8, 0.01 *M* NaCl. The homogenized tissue was strained through 4 layers of cheesecloth and the supernatant from a 5 min centrifugation at 200 × g was centrifuged again at 1.500 × g for 15 min. The precipitates were resuspended in 10 ml medium used for extraction and homogenized in a glass homogenizer. The deriving chloroplasts were used in a model system containing 0.02 mg chlorophyll, 40 μM Tris buffer, 0.068 μM 2,6-dichlorophenol-indophenol, 5 μM phosphate, 0.03 μM phenazin methosulphate, 10 μM magnesium ion. The methyl-benzimidazole carbamate (benomyl) was applied in an ethanolic solution (100 ppm).

Respiration rate of the leaf discs was measured by the standard Warburg technique (UMBREIT et al., 1959) at 25 °C. Isocitric dehydrogenase was measured in a mitochondrial suspension by the UV method of SIEBERT (1965) at 366 nm. The mitochondria were prepared by the procedure of PIERPOINT as was described elsewhere by KRISTEV (1964). 50 g cold leaf tissue was homogenized in a precooled mortar with 100 ml of medium containing 0.4 *M* sucrose, 0.05 *M* Tris buffer, pH 7.2 and 0.005 *M* EDTA. After straining the homogenate through 4 layers of cheesecloth, the resulting filtrate was centrifuged at 1.500 × g for 10 min. The supernatant was recentrifuged for 20 min at 10,000 × g and the pellet containing mitochondria was suspended in 50 ml medium used for extraction. After recentrifugation at 15,000 × g for 20 min the supernatant was discarded and the pellet suspended in 10 ml medium containing 0.3 *M* sucrose and 0.05 *M* Tris buffer (pH 7.2) in a glass homonenizer.

Procedures for the isolation of chloroplast or mitochondria were carried out at 0 – +4°C in a refrigerated centrifuge.

Results

Apparent photosynthesis. The time course of apparent photosynthesis for apple leaf discs used in the first experiment is shown in Fig. 1. From this it may be seen that the activity of apperent photosynthesis decreases in the leaf discs after spraying the leaves with benonyl. The reduction of apparent photosynthesis remains unchanged after the whole course of spraying.

Effect on the Hill reaction. The inhibition of the Hill reaction or photochemical reduction of water by chloroplasts is a well known effect of several herbicides (GOOD, 1961). The inhibitory effect of benomyl on the Hill reaction or its effect on the ability of isolated chloroplasts to reduce 2,6-dichlorophenol indophenol in the presence of phenazine-methosulphate in light is demonstrated in Fig. 2.

The optical density measured at 535 nm before and after the light reaction (and measured at every 30 sec) decreased in the controls twice as rapidly as in the presence of methyl-benzimidazole carbamate. In other words, the Hill reaction is inhibited in the chloroplasts by the benomyl.

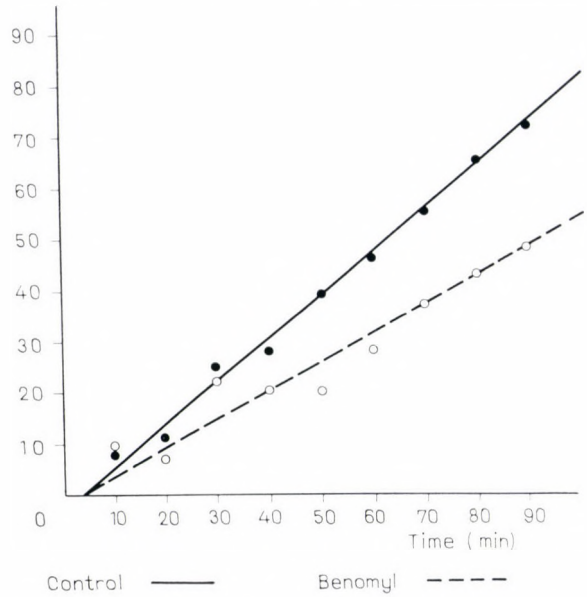

Fig. 1. Rate of apparent photosynthesis (μl/4 cm^2/hr)

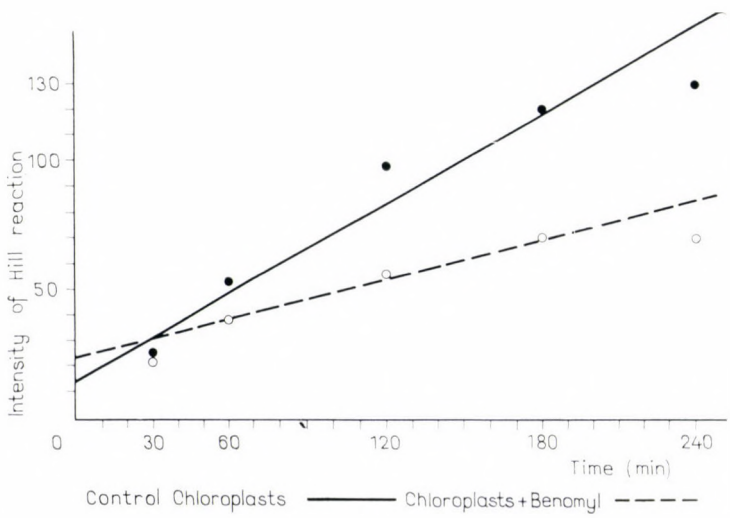

Fig. 2. Inhibition of Hill reaction by benomyl

This effect is closely correlated with a lower intensity of apparent photosynthesis in apple leaves sprayed with benomyl.

Respiratory rate. The data presented in Table 1 show the intensity of respiration of leaf discs from sprayed and unsprayed apple leaves.

Table 1

Respiratory rate of leaf discs of sprayed and control apple leaves

20 discs of apple leaves per flask	O_2-consumption		
	µl/mg N/hr	%	No. of determinations
Control (unsprayed)	135.82	100	9
Sprayed with benomyl	139.61	103	9

It may be seen that no change of respiratory rate in apple leaves sprayed with benomyl was found as calculated on the basis of mg nitrogen.

The activity of isocitric dehydrogenase. Activity of this enzyme was measured in a mitochondrial suspension. Isocitiric dehydrogenase was insensitive to benomyl in these experiments. One can conclude that the benomyl has no effect upon the Krebs tricarboxylic acid cycle located in mitochondria.

Discussion

One, two or three applications of the systemic fungicide benomyl to apple plants disturb apparent photosynthesis in the leaves significantly. In model experiments this fungicide inhibits almost 50 per cent of the intensity of Hill reaction in the chloroplasts.

On the other hand, benomyl has no effect on the intesity of respiration processes of apple leaves. Furthermore, we were unable to discover any changes in the activity of isocitric dehydrogenase in the mitochondria.

From these results it may be concluded that the benomyl is an inhibitor of photosynthesis in plant cells: it disturbes the Hill reaction in the chloroplasts and inhibits the photosynthetic activity in the leaf tissues. On the other hand, benomyl is not an inhibitor of the energy production processes in the plant cells: it does not disturb respiratory rate of tissues and also the key enzyme of the Krebs tricarboxylic cycle, isocitric dehydrogenase, remains unchanged.

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Experiments on the Fungistatic and Systemic Action of Benlate

By

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As a result of the tests conducted *in vitro* and *in vivo* with the systemic fungicide Benlate (Du Pont 1991) it was stated that 0.93 ppm water suspension gives a value of LD_{50} for the germination of spores of *Cladosporium cucumerinum*. In a poisoned food technique a 5 ppm concentration was inhibitory to the fungus. The effect of Benlate on powdery mildew and fusarial wilt diseases was also investigated. The systemic action of Benlate has been shown by paper chromatography using extracts from root, stem and leaves of plants treated with Benlate.

Benlate – methyl 1-butylcarbamoyl-2-benzimidazole carbamate, $C_{14}H_{18}N_4O_3$ – is available as a 50% wettable powder. It has been reported that Benlate was effectively applied against the following fungi: *Rhizoctonia solani*, *Venturia inaequalis*, *Fusarium* spp., *Erysiphe cichoracearum*, *Botrytis cinerea*, *Verticilium* etc., causing diseases in plants (AL-BELDAWI and PINCARD, 1968; ANONIMOUS, 1968; DELP and KLOPPING, 1968; ERWIN, MEE and SIMS, 1968).

This report summarizes the results of some experiments on the fungistatic and systemic action of Benlate. The experiments were carried out against *Cladosporium cucumerinum* and *Sphaerotheca fuliginea* of cucumber and *Fusarium oxysporum* f. *pisi* strains A, B, and C of pea.

The research was performed in the Phytopathology Laboratory at the Agricultural University, Wageningen, Holland (1969).

Materials and Methods

In vitro and *in vivo* techniques were used to evaluate the control of *Cladosporium cucumerinum* of cucumber by Benlate. *In vivo* techniques were used to evaluate the effectiveness of the fungicide against *Sphaerotheca fuliginea* of cucumber and *Fusarium oxysporum* f. *pisi* of pea.

In vitro experiments

The effect of Benlate on *C. cucumerinum* was determined *in vitro* using: a) A spore germination test and b) a poisoned food technique.

a) A spore germination technique was used to determine the LD 50. A spore suspension of a 7 days old pure culture of *C. cucumerinum* standardized at 200,000

spores per ml and suspensions of Benlate of various concentrations were used. For each concentration 8 drops were put on glass slides and incubated at 99% humidity.

b) In the poisoned food technique 9 ml Czapek-Dox agar, 1/2 ml spore suspension of *C. cucumerinum* (200,000 spores) and 1/2 ml Benlate suspension of varying concentrations were thoroughly mixed and put in a Petri dish. Each concentration was represented by two Petri dishes.

Table 1

Poisoned food technique to test *Cladosporium cucumerinum*

Control	0.16 ppm	0.32 ppm	0.63 ppm	1.25 ppm	2.5 ppm	5 ppm	10 ppm	20 ppm
+++	+++	+++	+++	+++	+	0	0	0

Note: +++ = germination on the whole field of a Petri dish.
++ = germination on the half field of a petri dish.
+ = several spots of germination on the Petri dish.
0 = no germination.

In vivo experiments

1. *C. cucumerinum.* Root absorption test. Roots of healthy 7 days old cucumber seedlings var. Lange gele Tros were placed in aqueous suspensions of various concentrations of Benlate (Table 2). After three days the plants were transferred to distilled water and the leaves inoculated with a spore suspension of *C. cucumerinum.*

Infection was assessed seven days after inoculation, according to a 0 to 4 scale (Table 2). Each treatment was represented by ten seedlings and included on appropriate control.

2. *S. fuliginea*

a) *Root absorption test.* Roots of healthy cucumber seedlings var. Groene with two to three leaves per plant were placed in aqueous suspensions of various concentrations of Benlate (Table 3). Two inoculation treatments were imposed with half the plants inoculated immediately after placing in the Benlate suspensions while the other half were inoculated three days later, when all plants of both treatments were transferred to water. Each concentration was replicated twice.

b) *Leaf disc test against S. fuliginea.* Leaf discs, cut from primary leaves of three weeks old cucumber plants var. Groene, were floated on aqueous suspensions of various concentrations of Benlate (Table 5) in open Petri dishes. Each concentration was represented by five discs. After three days the discs were transferred to water and the upper surface dusted with conidia of *S. fuliginea.* The discs were

Table 2

Effect of Benlate via roots to *Cladosporium cucumerinum*

Disease index	Number of plants					Mean disease index
	0	1	2	3	4	
Benlate						
0.32 ppm	—	—	—	1	9	3.9
0.63 ppm	—	—	—	1	9	3.9
1.25 ppm	—	—	—	3	7	3.7
2.50 ppm	—	1	4	3	2	2.6
5.00 ppm	7	2	1	—	—	0.4
10.00 ppm	10	—	—	—	—	0.0
20.00 ppm	10	—	—	—	—	0.0
Control	—	—	—	—	10	4.0

Marks: 0 = no *Cladosporium cucumerinum*
1 = 10% infection
2 = 10–50% infection
3 = 50–80% infection
4 = 80–100% infection

Table 3

Effect of Benlate via root to powdery mildew (Inoculation and immersion in the same day)

Disease index	Number of leaves							Mean disease index
	0	1	2	3	4	5	6	
Benlate								
0.32 ppm	—	2	—	—	—	2	2	4.0
0.63 ppm.	1	2	—	—	—	3	1	3.3
1.25 ppm	2	1	2	3	—	—	—	1.8
2.50 ppm	3	—	2	3	—	—	—	1.6
5.00 ppm	1	5	—	—	—	—	—	0.8
10.00 ppm	2	5	—	—	—	—	—	0.7
20.00 ppm	6	—	—	—	—	—	—	0.0
40.00 ppm	7	—	—	—	—	—	—	0.0
80.00 ppm	7	—	—	—	—	—	—	0.0
Control	—	2	—	—	—	2	4	4.5

Marks: 0 = no mildew; 1 to 6 = leaves covered with mildew.

incubated in the greenhouse at +20°C. Infection was estimated according to a 0–6 scale. Appropriate controls were included.

3. *F. oxysporum* f. *pisi*. The effectiveness of Benlate to control *F. oxysporum* f. *pisi* on peas var. Mansholt was determined using the following methods:

Table 4

Effect of Benlate via root to powdery mildew (Inoculation: 3 days after immersion in suspension)

Disease index	Number of leaves							Mean disease index
	0	1	2	3	4	5	6	
Benlate								
0.32 ppm	1	2	—	—	—	—	4	3.7
0.63 ppm	2	2	—	—	2	2	—	2.5
1.25 ppm	1	1	1	—	1	1	—	2.4
2.50 ppm	2	2	1	3	—	—	—	1.6
5.00 ppm	3	—	5	—	—	—	—	1.3
10.00 ppm	2	3	—	—	—	—	—	0.6
20.00 ppm	7	—	—	—	—	—	—	0.0
40.00 ppm	7	—	—	—	—	—	—	0.0
80.00 ppm	5	—	—	—	—	—	—	0.0
Control	—	2	—	—	—	2	4	4.5

Marks: 0 = no mildew; 1 to 6 = leaves covered with mildew.

Table 5

Effect of Benlate in the leaf disc test against powdery mildew

Disease index	Number of discs							Mean disease index
	0	1	2	3	4	5	6	
Benlate								
0.32 ppm	—	—	5	—	—	—	—	2.0
0.63 ppm	5	—	—	—	—	—	—	0.0
1.25 ppm	5	—	—	—	—	—	—	0.0
2.50 ppm	5	—	—	—	—	—	—	0.0
5.00 ppm	5	—	—	—	—	—	—	0.0
10.00 ppm	5	—	—	—	—	—	—	0.0
20.00 ppm	5	—	—	—	—	—	—	0.0
40.00 ppm	5	—	—	—	—	—	—	0.0
80.00 ppm	5	—	—	—	—	—	—	0.0
Control	—	—	—	—	—	—	5	6.0

Marks: 0 = no mildew; 1 to 6 = a leaf disc covered with mildew.

a) *Application of Benlate to soil.* Twenty-five ml aliquots of aqueous suspensions of various concentrations of Benlate (Table 6) were added to pots containing sterile sand inoculated with a one-week-old shake culture of strains A, B and C of *F. oxysporum* f. *pisi*. Each treatment was replicated twice with three plants per replicate.

Table 6

Effect of Benlate in sterile sand infested with *Fusarium oxysporum* f. *pisi*.

Disease index	Number of plants					Mean disease index
	0	1	2	3	4	
Benlate						
320 ppm	3	3	—	—	—	0.5
160 ppm	—	4	2	—	—	1.3
80 ppm	—	1	4	1	—	2.0
Control	—	—	—	1	5	3.8

Marks: 0 = no *Fusarium oxysporum;* 1 to 4 = all plants inoculated with *Fusarium oxysporum* f. *pisi*.

b) *Soaking seeds.* Pea seeds were soaked in aqueous suspensions of various concentrations (80, 160 and 320 ppm) of Benlate for thirty minutes and sown in sterile soil inoculated with a pure culture of *F. oxysporum* f. *pisi*. Three seeds per pot were used.

c) *Application to leaves.* Twenty-day-old pea plants growing in pots with sterile soil were sprayed with aqueous suspensions of various concentrations (40, 80, 160 and 320 ppm) of Benlate and the soil inoculated with *F. oxysporum* f. *pisi*. Each treatment was replicated twice with four plants per replicate.

Experiments to determine the effectiveness of Benlate against *F. oxysporum* f. *pisi* were conducted in a greenhouse at +25°C. Infection was assessed according to a 0–4 scale. In all cases appropriate controls were included.

Demonstration of transport of Benlate in the plant. For the study of the transport of the chemical in the plant we applied a paper chromatography technique. Chromatograms were made on Whatman 3 MM filter paper and developed with propanol: water 85 : 15 at +25°C. Fungitoxic compounds were demonstrated by a method cited by DEKHUIJZEN (1961) with *C. cucumerinum* as a test fungus.

1. Sap (60 microliter) from cucumber seedlings, that had been immersed with their roots in a 150 ppm suspension of Benlate for two days was compared to the sap of pure Benlate suspension (40 microliter) and to the sap from cucumber seedlings immersed with their roots in water for two days (60 microliter).

2. Sap was prepared from the roots, stem and leaves of seven days old cucumber seedlings, having been immersed in a 150 ppm suspension of Benlate for two days. 50 microliters of each sap were spotted on one chromatogram and the chromatograms developed during 14 1/2 hours in propanol : water 85 : 15, dried and sprayed with a spore suspension of *C. cucumerinum* (4,000,000 sp./ml).

Results

Experiments in vitro

a) *Spore germination test.* A LD 50 was obtained with 0.93 ppm Benlate suspension (inhibition of spore germination of *C. cucumerinum)* (Fig. 1).

b) *Poisoned food technique on agar.* A Benlate suspension of 5 ppm concentration inhibited the germination of spores of *C. cucumerinum* (Table 1).

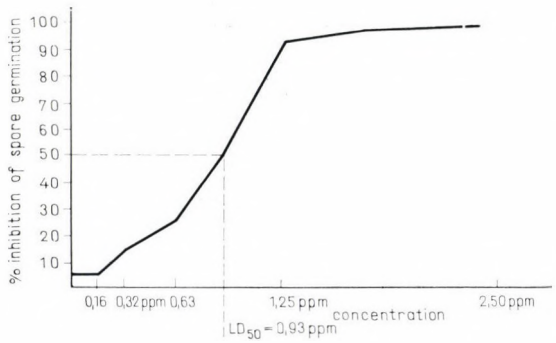

Fig. 1. The effect of Benlate on the germination of spores of *C. cucumerinum*

Experiments in vivo

1. *Via roots — against C. cumerinum.* Full protection was obtained with 10 ppm of Benlate. This gave a disease index of 0. With 5 ppm a disease index of 0.4 was obtained (Table 2).

2. *Root test against powdery mildew.* Using the two variants good protection was obtained with 20 ppm of Benlate suspension (disease index 0). With 5 ppm a disease index of 0.8 was obtained (see Table 3 and Table 4) (Fig. 2).

Using the leaf discs technique, full inhibition was obtained with a concentration of 0.63 ppm (see Table 5).

Phytotoxicity. The compound Benlate showed a tendency to be phytotoxic at 80 ppm. Light spots were observed along the margin and veins of the leaf.

On the leaf discs, floating on 80 ppm, light circles were observed at the disc margin.

Experiments with wilt disease

A good protection was obtained only by the application of the chemical to sterile sand using a concentration of 320 ppm of Benlate suspension (disease index of 0.5). (See Table 6 and Fig. 3).

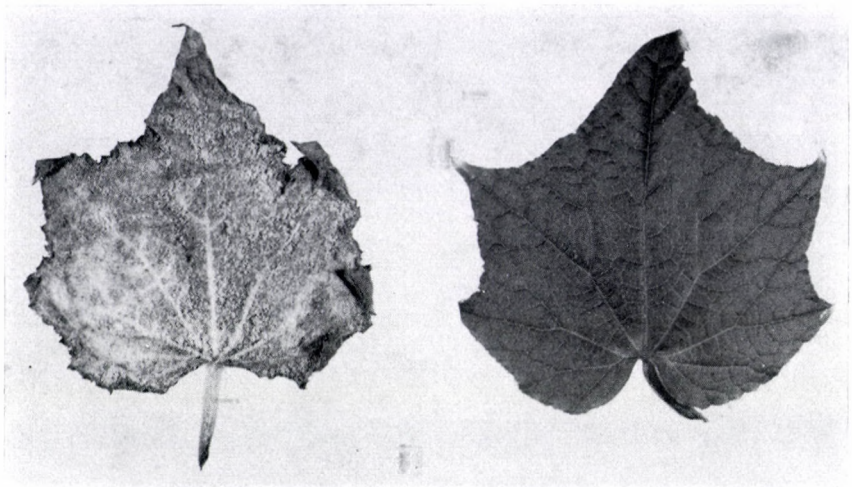

Fig. 2. The effect of Benlate on the powdery mildew of cucumber *(Sphaerotheca fuliginea)*. Left: control, soaked in water and infected. Right: leaf from a plant soaked in 20 ppm of Benlate and infected

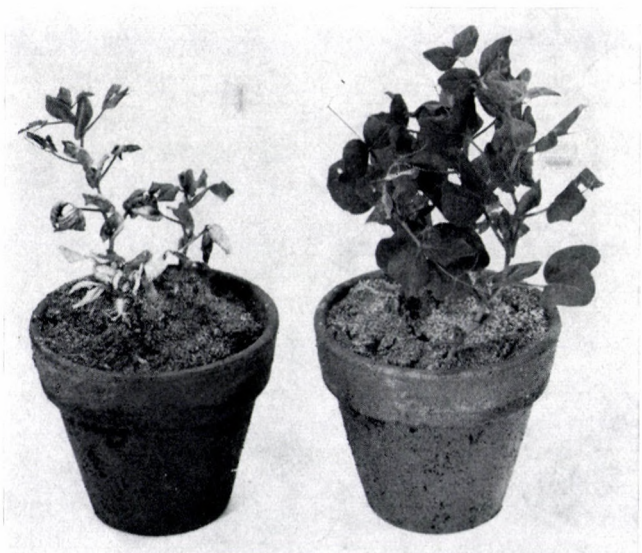

Fig. 3.The effect of Benlate on fusarial wilt of peas *(Fusarium oxysporum* f.*pisi)*. Left: control, 25 ml water to the soil. Right: 25 ml Benlate suspension (320 ppm) applied to the soil

With 160 ppm of Benlate a disease index of 1.3 was obtained. For the control the disease index was 3.8.

Experiment with the techniques of soaking seeds in the course of 30 minutes did not prevent the plants from infection.

When sprinkling the peay leaves with Benlate, no effective resistance was observed against *F. oxysporum* f. *pisi*. It can be presumed that the compound, when applied to the pea leaves, fails to produce any effect.

Transport of Benlate in the plant

For pure Benlate a R_f value of 0.81 was obtained. For the sap of cucumber, which had been immersed with their roots in 150 ppm of Benlate for two days, the R_f value was also 0.81. (Fig. 4).

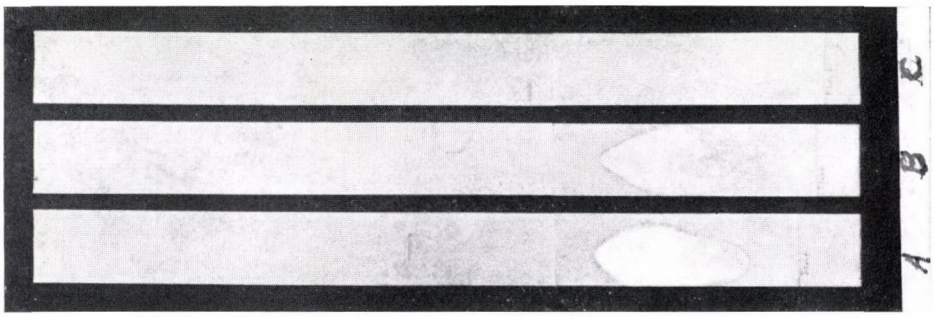

Fig. 4. Paper chromatographic determination of Benlate. A: 150 ppm Benlate in water. B: sap of cucumber seedlings immersed with their roots in 150 ppm of Benlate for 2 days C: roots immersed in water (control)

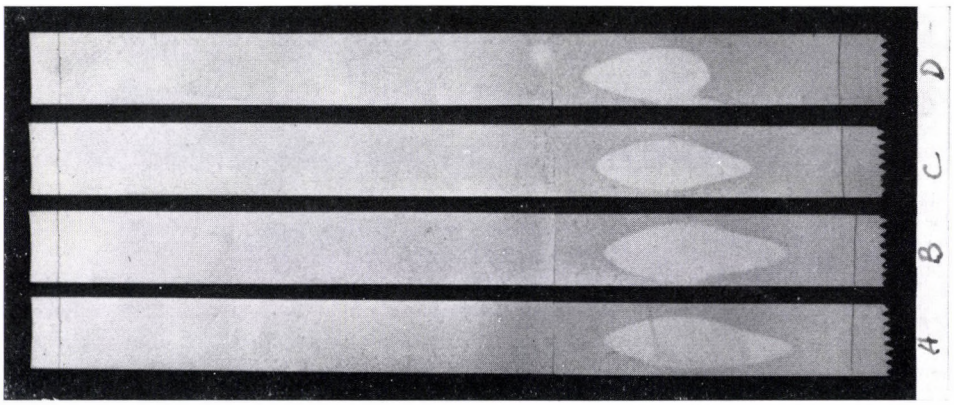

Fig. 5. Paper chromatographic determination of Benlate. A: in a 150 ppm water suspension, B: in root sap of cucumber seedlings immersed with their roots in 150 ppm of Benlate for 2 days, C: in the sap of stems, D: in leaf sap from seedlings similarly treated with Benlate

One can suppose that the similar R_f values obtained are related to one and the same compound: BCM (CLEMONS and SISLER, 1969).

From chromatographic experiments it was concluded that the active compound in the different parts of cucumber seedlings (roots, stems and leaves) was transported to the leaves after two days of immersion of the roots in a 150 ppm Benlate suspension.

On the chromatograms large white spots were formed where *C. cucumerinum* failed to develop. These spots had about similar R_f values (Fig. 5).

Literature

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Synthesis and Antifungal Action of Some New Dithiocarboxylic Acid Derivatives

By

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Several dithiocarboxylic acids, their esters and cyclic derivatives (benzothiazinethions) were prepared, based on the reaction of active methylene compounds with carbon disulfide and ammonia. These compounds contain functional groups serving as ligands for metal-chelates. Due to the role of the Cu-containing polyphenol oxidase in resistance, their contact and systemic antifungal activity have been tested. The results indicate that several representatives of this class act as contact fungicides with a moderate systemic action. The experimental data permits some conclusions on structure-activity relationship and on the biological significance of their chelating capacity.

While the antifungal properties of the dithiocarbamic acids were widely studied, few attention has been devoted to the dithiocarboxylic acids in this respect. This is due partly to the difficulties connected with their synthesis, partly to the fact that the nitrogenatom of the dithiocarbamates is generally regarded as a requirement of activity.

In the latest chemical literature some new dithiocarboxylic acids and their derivatives were mentioned which can be easily be obtained and which contain a nitrogen atom in their molecule. In a series of publications TAKESHIMA and co-workers have demonstrated that cyclopentanone reacts in the presence of ammonia with carbon disulfide to give 2-amino-1-cyclopentene-1-dithiocarboxylic acid (Fig. 1) [1], which can be converted by treatment with ketones into the thiazines [2, 3] and [4]. Cyclohexanone yields under similar conditions the dithiocarboxylic acid salt [5], which, when treated with an acidic reagent, is cyclised into thiono-pentahydro-benzothiazine-spiro-cyclohexane [6].

These compounds attracted our attention for several reasons. First, they include the structural fragments of the dithiocarbamic acids and can be regarded as vinilogs thereof. Secondly, they exist in several tautomeric forms. Results of Brown on the antifungal activity of rhodanines, as well as our own studies on α-oxo-methylene- and α,β-dioxo-compounds suggest that within certain groups of compounds the most actives are those providing the greatest possibility for tautomerisation.

The third, perhaps most emphatic reason why this type of compounds aroused our attention is the fact that their functional groups are known to act as ligands

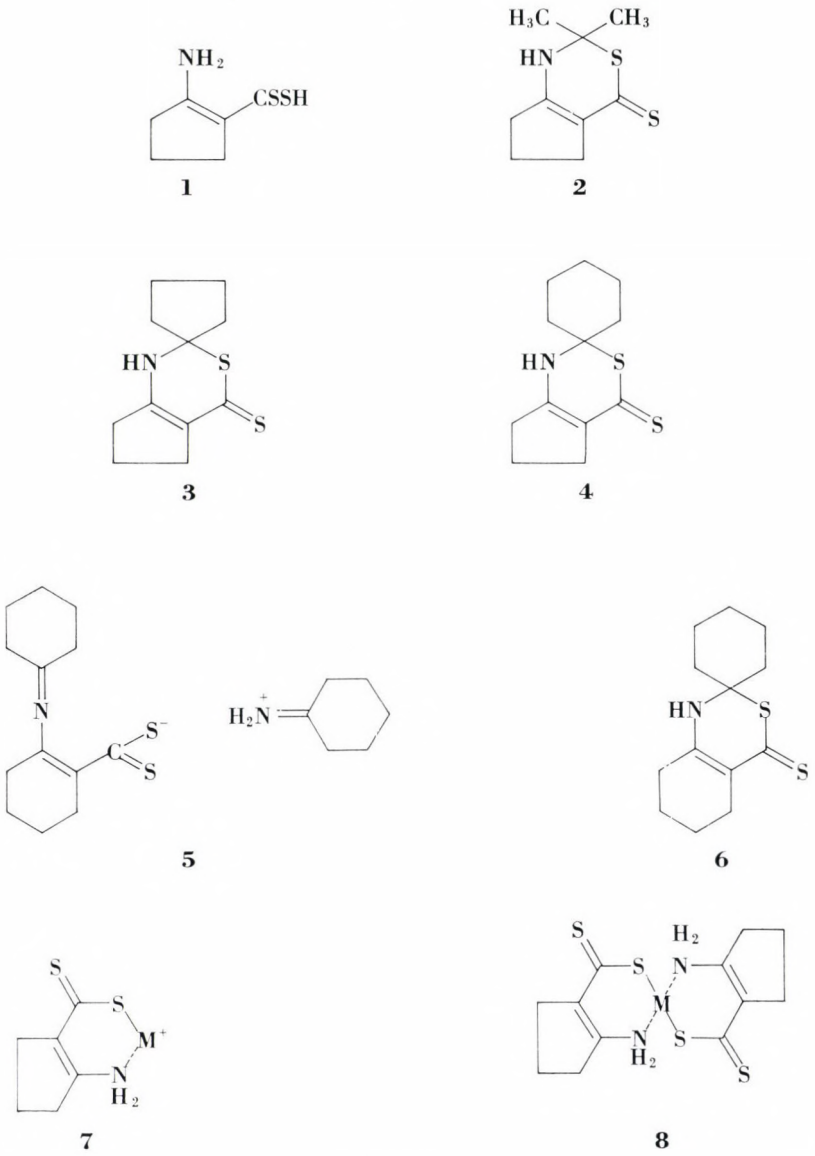


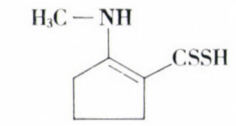
Fig. 1

in metal-complexes. Of special interest in this respect is compound [1], the functional groups of which are situated in the molecule so that they permit the formation of six membered chelates with a metal atom as closing member [7, 8]. Inactivation of metals, especially the selective inactivation of copper seems to be a prom-

ising approach for the selective combat of plant diseases. From the works of KAARS SIJPESTEIJN, VAN ANDEL and FUCHS it is known that inhibition of polyphenol oxidase, a copper-containing enzyme system plays an important role in the defence mechanism of plants, partly by causing lignification on penetration of the fungus, partly by stimulating indolylacetic acid oxydase. Another possibility to obtain antifungal effect is provided by the inhibition of lipoic acid by the copper carried into the cell by an appropriate complexing agent.

According to ALBERT complexing agents can act on two ways: either by inactivation of a metal in its biological setting or by increasing its local concentration.

The synthesis of several new derivatives within this type has been carried out by us. The compounds are shown in Fig. 2. Reacting cyclopentanone with carbon disulfide and methylamine yielded 2-methylamino-1-cyclopentene-1-dithiocarboxylic acid (Fig. 2) [9] in 22% yield, besides methylammonium methyldithiocarbam-



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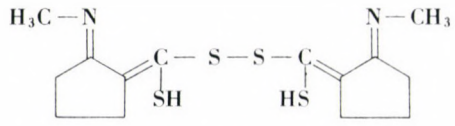

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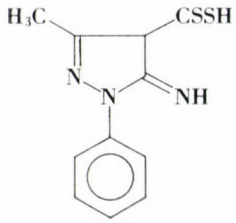

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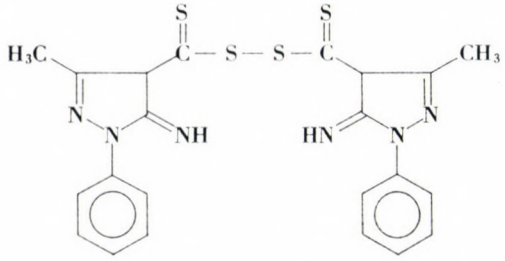

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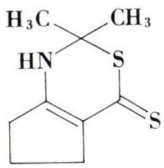
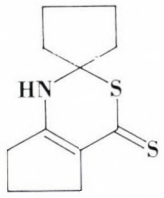
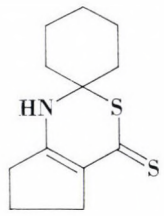
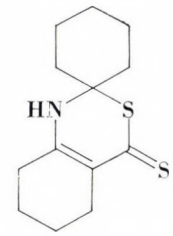
Fig. 2

ate as the main product. Other aliphatic and aromatic amines do not enter this reaction. On oxidation this compound yielded the disulfide [10]. We succeeded to obtain compound 9 also on a different way, by an equilibrium amine-exchange reaction. This last reaction can be applied also for the preparation of other N-alkyl-cyclopentene-dithiocarboxylic acids and their esters.

Our efforts to react carbon disulfide and ammonia with other five-membered active methylene compounds, such as rhodanines, hydantoins, isoxazolones, etc. failed, with exception of 3-methyl-1-phenyl-2-pyrazol-5-one, which yielded 5-imino-3-methyl-1-phenyl-2-pyrazoline-4-dithiocarboxylic acid [11]. This com-

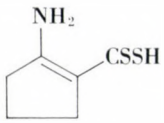
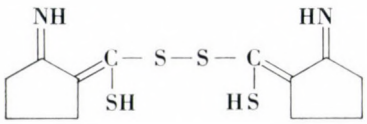
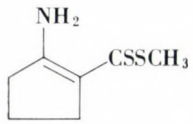
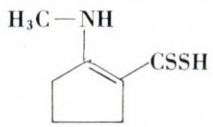
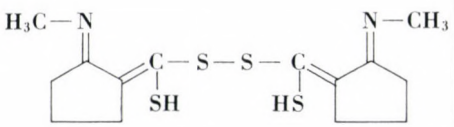
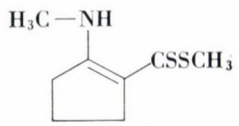
pound could be converted with cautious oxidation into the disulfide [12]. Methyl esters of these dithiocarboxylic acids, not shown on these figures, were prepared by routine methylation, using dimethylsulfate or diazomethane as methylating agent.

Table 1

		Alternaria tenuis		*Botrytis allii*	
		germination	growth	germination	growth
2	**2** 	$2 \cdot 10^{-2}$	$2 \cdot 10^{-3}$	10^{-2}	10^{-2}
3	**3** 	$6 \cdot 10^{-3}$	$2 \cdot 10^{-3}$	$7 \cdot 10^{-4}$	$3 \cdot 10^{-4}$
4	**4** 	10^{-2}	$7 \cdot 10^{-3}$	10^{-3}	$2 \cdot 10^{-3}$
6	**6** 	$2 \cdot 10^{-1}$	10^{-1}	10^{-1}	$2 \cdot 10^{-1}$
	zinc-dimethyl dithiocarbamate (control)	$6 \cdot 10^{-3}$	10^{-3}	$4 \cdot 10^{-3}$	$3 \cdot 10^{-4}$

The antifungal tests were carried out by the standard spore germination techniques of McCallan, modified by BÁNKI and co-workers. *Alternaria tenuis* and *Botrytis allii* were used as test organisms. The minimal concentration of the

Table 2

	Alternaria tenuis		*Botrytis allii*	
	germination	growth	germination	growth
1 	$6 \cdot 10^{-3}$	$3 \cdot 10^{-3}$	$5 \cdot 10^{-3}$	$2 \cdot 10^{-3}$
	10^{-2}	$5 \cdot 10^{-3}$	$9 \cdot 10^{-3}$	$7 \cdot 10^{-3}$
	$8 \cdot 10^{-3}$	$6 \cdot 10^{-3}$	$3 \cdot 10^{-2}$	$2 \cdot 10^{-2}$
9 	10^{-2}	$9 \cdot 10^{-3}$	$3 \cdot 10^{-3}$	10^{-3}
	$7 \cdot 10^{-2}$	10^{-2}	$4 \cdot 10^{-2}$	10^{-2}
	10^{-1}	$6 \cdot 10^{-2}$	10^{-1}	10^{-2}
zync-dimethyldithiocarbamate (control)	$6 \cdot 10^{-3}$	10^{-3}	$4 \cdot 10^{-3}$	$3 \cdot 10^{-4}$

compound, expressed in percentage, required for a 50% inhibition of germination and growth, respectively, served as measure of contact antifungal activity.

Leaf to leaf systemic activity was tested on beans *(Phaseolus vulgaris)* infected with *Uromyces appendiculatus* and on cucumber infected with *Erysiphe cychoracearum*. The chemicals were applied to portions of the foliage, assessing control both on the sprayed and on the unsprayed leaves.

Table 1 shows the contact antifungal activity of several thiono-thiazine derivatives. Compound [6] is negligibly fungitoxic. With gradual substitution of the folded six-membered rings for flat five-membered rings [4, 3] the activity gradually increases, reaching the same activity as ziram used as control. Elimination of the spiro-structure [2] results a medium activity within this series.

Table 2 shows the comparative antifungal activity of some amino-cyclopentene-dithiocarboxylic acids and their derivatives. The activity of the N-methyl-derivative [9] is somewhat less than that of the unsubstituted amino compound [1]. Esterification of both acids resulted a drop of activity, similarily to the decreased activity of the dithiocarbamic acid esters as compared to their salts. Also the disulfides are somewhat less phytotoxic than their parent acids.

Leaf to leaf systemic antifungal action of compounds [1] and [9] is shown on Table 3. The compounds were tested on two host—parasite combinations in 200 ppm. concentration. Both dithiocarboxylic acids showed a moderate systemic

Table 3

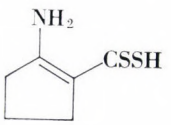
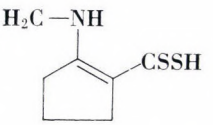
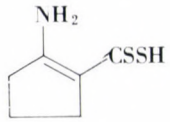
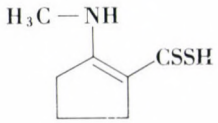
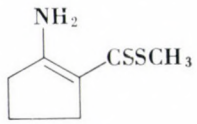
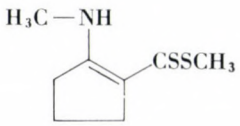
	Host-parasite combination	Systemic activity (leaf to leaf) in % of untreated control					
		3	6	9	12	15	18
		days after infection					
1 	Phaseolus vulgaris + Uromyces appendiculatus	—	—	—	—	—	—
	Cucumis sativus + Erysiphe cichoracearum	100	90	50	22	—	—
9 	Phaseolus vulgaris + Uromyces appendiculatus	100	100	90	80	—	—
	Cucumis sativus + Erysiphe cichoracearum	100	90	50	30	20	—

Table 4

		Chelating capacity	Antifungal activity	Polyphenol-oxidase inhibition p.p.m.
1		high	++++	10
9		medium	+++	>100
		low	++	10—100
		low	+	>100

activity. Benomyl, applied as a control substance, gave under similar conditions 100% protection even after 15 days.

Due to the possible function of these compounds as inhibitors of polyphenol oxidase, their polyphenol oxidase inhibiting activity was tested by using the dihydroxy-phenylalanine test and the results were paralleled with the antifungal data. The results are shown in Table 4. Compound [1] possesses the highest chelating capacity; in the meanwhile it shows the highest antifungal action and the strongest polyphenol oxidase inhibition. With gradual decrease of the chelating capacity both antifungal action and inhibition of polyphenol oxidase are lowered.

In our next set of experiments we have tested the influence of complex formation on the antifungal activity of the compounds under investigation. We prepared their 1 : 1 ionic complexes formed with copper, mercury and zinc, the

first as representative of a metal present, the other two as of metals absent in enzymes. This was based on the well-known finding that heavy metal enzymes are inhibited by metal chelates where the chelated metal is different from that of the enzyme.

The data presented on Table 5 show no pronounced difference neither between the absolute effectivity of the two metal complexes, nor as regards to the

Table 5

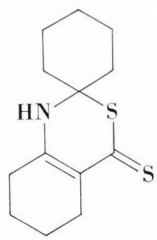
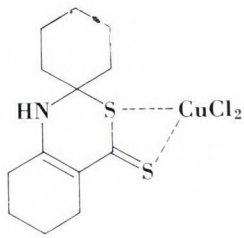
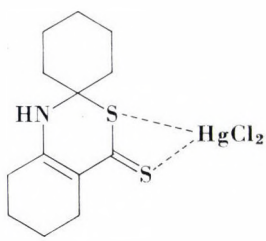
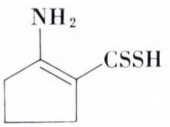
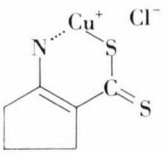
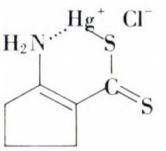
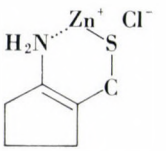
	Alternaria tenuis		*Botrytis allii*	
	germination	growth	germination	growth
 **6**	$2 \cdot 10^{-1}$	10^{-1}	10^{-1}	$2 \cdot 10^{-1}$
	$5 \cdot 10^{-3}$	10^{-3}	$5 \cdot 10^{-3}$	$2 \cdot 10^{-3}$
	10^{-3}	$3 \cdot 10^{-4}$	$3 \cdot 10^{-3}$	$7 \cdot 10^{-4}$
$CuCl_2$ (equimol.)	$2 \cdot 10^{-4}$	$9 \cdot 10^{-5}$	$2 \cdot 10^{-3}$	$2 \cdot 10^{-4}$
$HgCl_2$ (equimol.)	$2 \cdot 10^{-4}$	$5 \cdot 10^{-5}$	$8 \cdot 10^{-4}$	$2 \cdot 10^{-4}$

Table 6

	Alternaria tenuis		*Botrytis allii*	
	germination	growth	germination	growth
 I	$6 \cdot 10^{-3}$	$2 \cdot 10^{-3}$	$5 \cdot 10^{-3}$	$2 \cdot 10^{-3}$
	$2 \cdot 10^{-2}$	10^{-2}	$7 \cdot 10^{-2}$	$2 \cdot 10^{-2}$
	$2 \cdot 10^{-2}$	$5 \cdot 10^{-3}$	$3 \cdot 10^{-2}$	$9 \cdot 10^{-3}$
	$6 \cdot 10^{-1}$	$6 \cdot 10^{-1}$	$2 \cdot 10^{-2}$	$4 \cdot 10^{-3}$
$CuCl_2$ (equimol.)	$2 \cdot 10^{-4}$	$9 \cdot 10^{-5}$	$2 \cdot 10^{-3}$	$2 \cdot 10^{-4}$
$HgCl_2$ (equimol.)	$2 \cdot 10^{-4}$	$5 \cdot 10^{-5}$	$8 \cdot 10^{-4}$	$2 \cdot 10^{-4}$
$ZnCl_2$ (equimol.)	$8 \cdot 10^{-3}$	$5 \cdot 10^{-3}$	$6 \cdot 10^{-3}$	$2 \cdot 10^{-3}$

rate of their activity to the corresponding inorganic metal salt. Both complexes were more active than the uncomplexed ligand but were less effective than equivalent amounts of the corresponding inorganic metal salts.

Also the metal complexes of 2-amino-1-cyclopentene-1-dithiocarboxylic acid were less active than the uncomplexed metals, as shown by the data of Table 6. Even the original activity of the uncomplexed ligand has been sharply decreased as a result of chelation.

Table 7

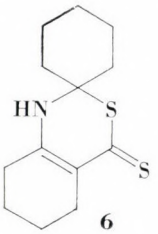
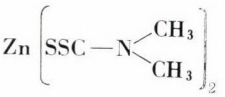
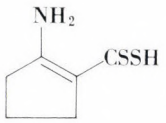
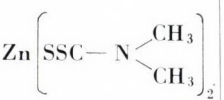
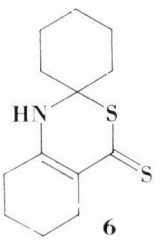
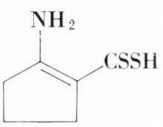
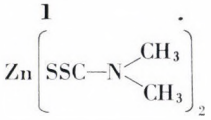
	Alternaria tenuis		*Botrytis allii*	
	germination	growth	germination	growth
 **6** + 	$7 \cdot 10^{-3}$	$3 \cdot 10^{-3}$	$<10^{-6}$	$<10^{-6}$
 **1** + 	$7 \cdot 10^{-3}$	10^{-3}	$<10^{-6}$	$<10^{-6}$
 **6**	$2 \cdot 10^{-1}$	10^{-1}	10^{-1}	$2 \cdot 10^{-1}$
 **1** + 	$6 \cdot 10^{-3}$	$3 \cdot 10^{-3}$	$5 \cdot 10^{-3}$	$2 \cdot 10^{-3}$
	$6 \cdot 10^{-3}$	10^{-3}	$4 \cdot 10^{-3}$	$3 \cdot 10^{-4}$

Table 7 presents the results of experiments where the joint action of the investigated compounds and of zinc dimethyldithiocarbamate was studied. Comparing the antifungal activity of a mixture of compound [6] and zinc dimethyldithiocarbamate with the activity of either of the two components, it is apparent that while

the joint action of the two compounds against *Alternaria* corresponds to the additive action, the activity against *Botrytis* is potentiated enormously.

The reason of this phenomenon is obscure but its different appearance depending on the fungus species restricts the range of possible explanations to a mechanism which is present in one of the two fungi and absent in the other. The studies of FISHER and RICHMOND in Long Ashton may throw some light on our finding. By means of electrophoretic mobility studies they demonstrated that the spore surfaces both of *Botrytis* and of *Alternaria* contain amino and carboxylic groups originated from proteins. There is much similarity even in the set of amino acids of the two fungi, lysine, histidine and leucine being present in both. One of the few differences between the fungi, however, is that while *Alternaria* contains tyrosine, *Botrytis* does not. Our finding that the potentiation of the dithiocarbamate action takes place against *Botrytis* only but not against *Alternaria*, might be connected with the presence of tyrosine in *Alternaria*. The tyrosine fragments of the proteins might antagonise this potentiation on some or other way, perhaps by binding the active molecule to an inactive site of the protein surface. In the early fifties KLOTZ and co-workers, while studying the interactions of organic dyes with proteins, came to the conclusion that a protein tyrosine is often involved in this interaction, by binding the nitrogen atom of the dye molecule by hydrogen bond while zinc can increase the binding, forming a bridge between the protein and the compound by chelation.

Experiments are in progress to test whether the potentiation against *Botrytis* can be counterbalanced by simultaneous application of tyrosine. Further studies, also being in progress, are aimed to test whether the antifungal activity of the ethylene-bisdithiocarbamates can be potentiated on the same way. From the works of VAN DER KERK and co-workers it is known that in spite of the chemical similarities between dialkyldithiocarbamates and alkylenebisdithiocarbamates, their mode of action rests on different basis. It can be hoped therefore that elucidation of this finding will present useful information also as regards to the mechanism of action of the dithiocarbamates.

As concluding remark I wish to apologise for presenting mainly non-systemic fungicide studies on a symposium devoted to systemic fungicides. I think however that this is partly justified by the fact that we do not treat these compounds merely as representatives of a new class of fungicides but also as model compounds for the study of various mechanisms of antifungal effect working within the plant tissues.

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Studies on Chemical Control of *Ophiobolus graminis* Sacc.

I. Testing Systemic Fungicides against *Ophiobolus graminis* Sacc.

By

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Seven new systemic fungicides: Plantvax, Vitavax, Milcurb, Milstem, Voronit, Benlate and Minocol were tested as seed dressings against *Ophiobolus graminis*. *In vitro* inhibition of mycelial carried out using Benlate and Vitavax. In greenhouse experiments only Benlate gave a satisfactory result of disease control in a rate of 400 g/100 kg of seed.

Ophiobolus graminis Sacc. (*Gäumannomyces graminis* Arx et Olivier) is the pathogen of take-all disease of wheat. This disease is widely spread on Polish wheat fields (MICHALSKI and GORSKA-POCZOPKO, 1958; GORSKA-POCZOPKO, 1963) and although its occurrence-gradient is rather low (1 – 2 per cent [GORSKA-POCZOPKO, 1963]) due to its very severe harmfulness-coefficient of about 75 per cent (GORSKA-POCZOPKO, 1961) losses in crops have a substantial economic significance (GORSKA-POCZOPKO, 1962).

The disease is well-known in many countries of Europe, America, Asia, Africa and Australia.

Recently, we could find some new information on the occurrence of *Ophiobolus graminis* in India (GHURDE, 1967), Yugoslavia (KOSTIC, SMIJANKOVIC and TESLIC, 1966), Roumania (SAVULESCU and PZSCASU, 1967), Hungary (LELLEY, 1967) and new places in the USSR (GAVRILOV, 1968; KUZNIECOVA, 1966). The control of the take-all disease of wheat, due to the biology of the pathogen, is a hard problem. The best known control measure is crop rotation. Recently, some new papers appeared dealing with this problem (LEMAIRE and COPPENET, 1968; BOCKMANN, 1968; DEFOSSE and RIXHON, 1968; SLOPE, 1968).

Some authors recommended also nitrogen fertilization (HUBER, PAINTER, MCKAY and PETERSON, 1968; ROSSER and CHATBURN, 1965) to control the take-all disease. It seems, however, that its effect is mostly due to stimulation of host development rather than to the direct control of the pathogen. Nitrogen fertilizers according to SCOTT prolonged *Ophiobolus graminis* survival in buried wheat straw (SCOTT, 1969).

Attempts were also made to test various chemicals used as seed dressing in control of *Ophiobolus graminis*. KUZNIECOVA (1966) using mercury seed dressings

(Granosan, Merkuran) and tetramethyl thiuram disulphide alone or with hexachlorobenzene obtained positive results in controlling wheat diseases caused by *Fusarium*, *Ophiobolus* and *Helminthosporium*. SCHUMANN (1967) obtained best results in pot experiments with zineb although the field experiments did not confirm these results.

It is known that the main source of infection of *Ophiobolus graminis* is the soil, infested by resting mycelium of the fungus, remaining in wheat stubble and straw. Ascospores never were considered as an important source of infection because of a short period of their vitality.

Under Polish conditions it seems also not likely that the ascospores may play some important role because their formation is not regular (GORSKA-POCZOPKO, 1964). Young seedlings are especially susceptibile to *Ophiobolus graminis* during the first weeks of their development.

Ordinary seed dressing with non-systemic chemicals may only disinfect superficially the surface of the seed and partially sterilize the thin layer of soil around the seed.

Chemical dressings must possess the following properties:

a) abilities to penetrate into seed and thereafter into young plants of wheat (systemic activity),

b) antifungal activity against *Ophiobolus graminis*. This activity can be due to the fungicide or its metabolites in wheat plants.

The systemic fungicides in question fullfilled the condition "a" (penetration). Their antifungal activity against *Ophiobolus graminis* (condition "b") was not investigated so far. They possessed antifungal activity against other fungi (e.g. Vitavax against smuts, Plantvax against rusts, Milstem against powdery mildews). Some were also active against soil fungi (ALLAM, et al. 1969).

The aim of this work was to investigate the activity of some new systemic fungicides in controlling *Ophiobolus graminis* Sacc.

Materials and Methods

Own isolate of *Ophiobolus graminis* Sacc. was used in this study. Inoculum for laboratory as well as for greenhouse studies was prepared by subculturing the fungus on potato dextrose agar (PDA) prepared by using standard techniques and fresh potatoes.

The following fungicides were used:

1) Vitavax – Uni Royal's 5,6-Dihydro-2-methyl-1,4-oxatiin-3-carboxyanilide. Wettable powder, 75% active ingredient.

2) Plantvax – Uni Royal's 2,3-Dihydro-5-carboxanilido-6-methyl-1,4-oxathiin-4,4-dioxide. Wettable powder, 75% active ingredient.

3) Benlate – du Pont's 1991, 1-butyl-carbamoyl-2-benzimidazole carbamic acid methyl ester. Wettable powder, 50% active ingredient.

4) Milcurb-(ICI PP 675, methirimol) 5-Butyl-2-dimethylamino-4-hydroxy-6-methylpyrimidine. Formulation JF 2587 (solution of 125 g methirimol per litre).

5) Milstem – (ICI PP 149, ethirimol) 5-Butyl-2-ethylamino-4-hydroxy-6-methylpyrimidine. Formulation JF 2410, W. P. 80% active ingredient, powder.

6) Voronit – (Bayer's furidazole) 2/2'-furylo/-benzimidazole. Dry seed dressing of 3% furidazole and 20% hexachlorobenzene.

7) Minokol – Sumitomo S-45862. Composition unknown. Wettable powder, 75% active ingredient.

Laboratory studies

Culture plates were made of sterile PDA agar mixed with fungicides investigated. After solidifying the agar gel each plate was inoculated in five places with PDA discs containing mycelium of the test fungus *Ophiobolus graminis* and incubated in an incubator at the temperature of 20–22°C. Radial growth of the fungus was measured after 5–6 days and the inhibitory concentration determined.

Greenhouse studies

Wheat seed (*Triticum aestivum* var. Ostka Popularna) were used as non-treated (control) or as treated with fungicides (dry treatment or soaking). Dry seed treatment was performed in glass containers by shaking seed with fungicide during 10 minutes. Two levels of each fungicide were used namely 200 and 400 g of formulated material per 100 kg of seed.

Soaking treatment was performed in 0.3 per cent water suspension (or solution) of each fungicide for 24 hours. Seeds were then thoroughly cleaned by rinsing under tap water for 10 minutes and dried on filter paper.

After the treatment seeds were hand-planted in sand in paper pots (twenty five in each pot). Three pots were used for each combination as replications.

The sand was previously inoculated by the following way: twenty five holes 1 cm deep, 0.8 cm wide were made on the surface of sand with glass rod. On the bottom of each hole one agar disc, penetrated by the mycelium of *Ophiobolus graminis*, was put and one wheat kernel was located immediately on its surface. The seedlings were then incubated under normal greenhouse conditions for more than 3 weeks. During this time seedlings in the "control" became infected in about 100%.

Plants were then removed from sand, the roots rinsed in water, and the healthy and diseased plants were counted and per cent of diseased seedlings (y) was calculated according to the formula:

$$y = 100 \cdot \frac{\text{diseased seedlings in pot}}{\text{total amount of seedlings in pot}}$$

The length of seedlings was also measured and other observations were made in order to check phytotoxicity.

Preliminary greenhouse experiments were carried out in 1969 and were repeated twice in spring and summer of 1970, with very similar results.

Results and Discussion

The results of laboratory experiments (Table 1) clearly show the differences between the activity of fungicides against *Ophiobolus graminis* .The best inhibition of the growth on agar gave Benlate and Vitavax. Plantvax which is closely related to Vitavax oxatiine derivate, was almost inactive against *Ophiobolus graminis*. The pyrimidine derivates (Milcurb and Milstem) showed rather limited activity but the first was better than the second. Minocol was inactive.

In greenhouse experiments using dry seeds treatment (Table 2) Benlate showed a remarkable, statistically confirmed activity, especially at the level 400 g/100 kg seed, followed by Vitavax, Plantvax and Milcurb. Using lower level of fungicides, 200 g/100 kg seed only Benlate gave satisfactory control of take-all disease.

Similar patterns were found by soaking treatment (Table 3). Benlate again gave almost complete control of the disease. Vitavax and Milcurb were very weak in action, other fungicides were inactive. Milcurb induced some disturbances in seed germination.

The successful control of take-all disease in greenhouse experiments using Benlate, could be explained by its high *in vitro* activity. It does not explain, however, limited control obtained using Vitavax, which *in vitro* showed an activity almost as good as that of Benlate and is by itself a good systemic fungicide.

Table 1

Inhibition of mycelial growth of *Ophiobolus graminis* on potato dextrose agar mixed with systemic fungicides

	Fungicide	Common name of active ingredient	Minimal inhibitory concentration in ppm of active ingredient
1.	Vitavax	carboxin	3
2.	Plantvax	oxycarboxin	300
3.	Milcurb	methirimol	50
4.	Milstem	ethirimol	320
5.	Benlate	benomyl	1
6.	Voronit	furidazol + hexachloro-benzene	100
7.	Minokol	unknown	2.000

This phenomenon requires an explanation in further studies. Activity of Benlate in controlling take-all disease will also require confirmation in field experiments.

Table 2

Control of take-all disease of wheat seedlings in greenhouse experiments by dry seed treatment with fungicides

N°	Fungicide	Common name and per cent of active ingredient	Per cent diseased seedlings		Mean length of seedlings	Notes
			Level 200 g/100 seeds	Level 400 g/100 seeds		
1	Vitavax	carboxin 75%	69.8	47.7	27.7	
2	Plantvax	oxycarboxin 75%	74.0	53.4	28.3	
3	Milcurb	methirimol 12.5%	81.1	47.2	29.2	Germination disturbance
4	Milstem	ethirimol 80%	76.6	72.7	29.4	
5	Benlate	benomyl 50%	14.5	2.1	29.1	
6	Voronit	furidazole 3% + hexachlorobenzene 20%	73.3	55.2	28.9	
7	Minokol	unknown 50%	95.6	79.4	28.9	
—	Untreated	—	100.0	100.0	28.9	
Confidence interval 95%			21.0			
		99%	28.3			

Table 3

Control of take-all disease of wheat seedlings in greenhouse experiments with soaking the seeds in 0.3 per cent suspensions of the fungicides

N°	Fungicide	Common name and per cent of active ingredient	Per cent of diseased seedlings	Mean length of seedlings	Notes
1	Vitavax	carboxin 75%	61.6	28.4	
2	Plantvax	oxycarboxin 75%	77.9	28.3	
3	Milcurb	methirimol 12.5%	57.3	30.0	Germination disturbance
4	Milstem	ethirimol 80%	73.6	28.0	
5	Benlate	benomyl 50%	4.4	28.6	
6	Voronit	furidazol 3% + hexachlorobenzene	77.4	28.3	
7	Minokol	unknown 50%	84.5	28.4	
	Untreated	—	100.0	28.4	
Confidence interval — 95%			18.36		
		— 99%	24.75		

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Untersuchungen über die Entwicklung einer Toleranz bei phytopathogenen Pilzen gegenüber Systemfungiziden

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Species of five phytopathogenic fungi *(Pythium debaryanum, Sclerotinia sclerotiorum, Botrytis cinerea, Fusarium solani* f. *pisi, Rhizoctonia solani)* were cultivated in repeated passages on agar with additions of protective fungicides (dithiocarbamates, TMTD, captan and chlornitrobenzenes) and systemic fungicides (oxathiin-derivatives, thiabendazol). From the parent type of *F. solani* f. *pisi* a selection of resistant forms was given easier to systemic fungicides than to protective ones. In *S. sclerotiorum* it appeared resistant forms both to systemic and protective fungicides. In the case of other fungal species development of resistance to fungicides was not experienced. The selected resistant forms retained their behaviour of tolerance after several cultivations in the absence of fungicids, too. The resistance of a fungus to one fungicide was connected in a specific manner to tolerances to other fungicides. Tests on the pathogenicity revealed changes only in the resistant forms. It was shown in plant infection experiments that a regular application of a respective fungicide was not able to control the resistant form.

Aus der Prüfung wie auch Anwendung von Fungiziden ist bekannt, daß innerhalb ein- und derselben Pilzart Formen existieren, die sich in der Reaktionsnorm oder Empfindlichkeit gegenüber gleichen fungiziden Wirkstoffen beträchtlich unterscheiden (Strecker 1957, Thomas 1962, Shatla und Sinclair 1962, 1963, 1964, Chancogne und Fruchard 1965, Raffray und Sinclair 1965, Karg 1965, Savulescu et al. 1967, Reber 1967, Valaskova 1968, Mayer 1969, Luc et al. 1970). Die Fungizidempfindlichkeit eines Pilzes unterliegt demnach einer nicht unbedeutenden Variabilität, die bei einem entsprechenden Selektionsdruck, wie ständig einseitiger Verwendung von bestimmten Fungiziden, zur Entwicklung resistenter oder toleranter Populationen führen kann. Über die Möglichkeiten einer derartigen Entwicklung von Toleranzen bei phytopathogenen Pilzen gegenüber Fungiziden liegen bereits zahlreiche Untersuchungsergebnisse vor. Nach unseren Übersichten (Luc und Sung 1970) wurden bei mehr als 20 Pilzarten in über 60 Fällen gegenüber anorganischen oder organisch-synthetischen Wirkstoffen und z. T. auch Antibiotika Erscheinungen einer Resistenz- oder Toleranzbildung beobachtet. Überwiegend handelt es sich jedoch dabei um Toleranzen, die sich unter Laborbedingungen nach wiederholten Passagen der Pilze auf fungizidhaltigen Nährböden herausstellten. In weitaus geringerem Maße – nämlich nur bei 8

Pilzarten gegenüber bestimmten Fungiziden – liegen dagegen Ermittlungen über Toleranzerscheinungen unter natürlichen Verhältnissen vor. Sie beziehen sich ausschließlich auf Toleranzen gegenüber klassischen, d. h. protektiv wirkenden Fungiziden. Die offensichtlich geringere Gefahr einer potentiellen Fungizidtoleranzentwicklung in der Praxis, zu der vergleichsweise leichten Induktion fungizidtoleranter Pilzformen im Laboratorium, dürfte ihre Ursache einmal in der Spezifik ökologischer Bedingungen haben, die für eine Toleranzbildung von erheblicher Bedeutung sind (PARRY und WOOD, 1958, 1959, MCINTOSH, 1961, ELSAID und SINCLAIR, 1963, 1964, RAFFRAY und SINCLAIR, 1965, JANITOR und MAJERNIK, 1967). Zum anderen erwies sich aber auch, daß die fungizidtoleranten Formen im Vergleich zu den Normaltypen der Pilze oft eine verminderte Pathogenität und eine geringere Sporulationsfähigkeit und Wachstumsfreudigkeit aufwiesen, womit sie sich unter natürlichen Bedingungen nur wenig behaupten können (SISLER und COX, 1960, ASHIDA, 1965, GEORGOPOULOS und ZARACOVITIS, 1967).

Mit der zunehmenden Orientierung auf neuartige Systemfungizide entsteht nun die Frage, ob diese Vorstellungen über die praktische Bedeutung einer Toleranzentwicklung bei phytopathogenen Pilzen, wie sie mit der Verwendung der Protektivfungizide gewonnen wurden, weiterhin auch bei Systemfungizidverbindungen beibehalten werden können. Von diesbezüglichen Untersuchungen liegen bisher kaum Ergebnisse vor. DEKKER (1968), der sich mit den speziellen Mechanismen einer Fungizidresistenz bei Pilzen befaßte, hebt jedoch bereits hervor, daß gegenüber den weitaus spezifischer über den Stoffwechsel wirksamen Systemfungiziden, die Möglichkeiten des Herausbildens toleranter Formen größer sind. Um hierüber Anhaltspunkte zu gewinnen, führten wir vergleichende Untersuchungen über die Möglichkeit einer Selektion fungizidtoleranter Formen bei verschiedenen phytopathogenen Pilzarten gegenüber Protektiv- und Systemfungiziden durch und überprüften das weitere Verhalten der toleranten Typen hinsichtlich der Beständigkeit ihrer Fungizidtoleranz, auch gegenüber anders gearteten Wirkstoffen (Kreuzresistenz oder Kreuztoleranz), sowie ihr Betragen als Phytopathogene und ihre Bekämpfbarkeit in Infektionsexperimenten.

Material und Methoden

Zu den Versuchen wurden folgende Pilzarten herangezogen: *Pythium debaryanum* Hesse, *Fusarium solani* f. *pisi* (Jones) Snyd. et Hans., *Sclerotinia sclerotiorum* (Lib.) de Bary, *Botrytis cinerea* Pers. und *Rhizoctonia solani* Kühn.

An Protektivfungiziden verwendeten wir in Form von Handelspräparaten Zineb ('bercema Zineb 80'), Thiuram (TMTD, 'Wolfen Thiuram 85'), Captan ('Malipur'), Trichlordinitrobenzol (TCDNB, 'Olpisan') und Pentachlornitrobenzol (PCNB, 'Phomasan'), während an Systemfungiziden DCMO ('Vitavax'), DCMOD ('Plantvax') und Thiabendazol benutzt wurden. Als Kultursubstrat für die Pilze diente in Petrischalen einheitlich 2%iger Biomalzagar mit einem pH-

Wert von 5.9 . . . 6.1. Alle zu testenden Fungizide gaben wir flüssig in verschiedenen Aufwandmengen, die jeweils in ppm AS angegeben sind, kurz vor Erkalten des Agars zu. Die nachfolgende Beimpfung der Platten geschah durch ein 7 mm Agarimpfstück mit jungem Pilzmyzel einer entsprechenden Vorkultur. Nach Aufstellen der Platten bei 24 . . . 26 °C wurde täglich über eine Standard-Zeit von 10 Tagen das radiale Wachstum der Pilze gemessen. Unter Ausschaltung der lag-Phase errechneten wir die durchschnittliche tägliche Wachstumsrate und drückten im Vergleich zu einer fungizidfreien Kontrollvariante die Wuchsdifferenz eines Pilzes bei Einwirkung von Fungiziden als %ualen Hemmungsgrad bzw. als Wirkungsgrad des Fungizides aus. Dieser Wert wurde allen Beurteilungen und varianzanalytischen Berechnungen zugrundegelegt. Sämtliche Tests liefen in mindestens 4facher Wiederholung.

Im ersten Versuchsabschnitt wurden zunächst alle Pilzarten zur Selektion toleranterer Formen in 8 unmittelbar aufeinanderfolgenden Passagen auf Agarkulturen mit bestimmten Zusätzen von Protektivfungiziden oder Systemfungiziden getestet. An Fungizidaufwandmengen wählten wir dabei nach den von uns vorher ermittelten Dosis-Wirkungskurven für die einzelnen Pilzarten jeweils Dosierungen mit gutem, mittlerem und geringem Effekt aus. Die 8 Passagen erfolgten so, daß sowohl bei den fungizidfreien Kontrollen als auch bei den Fungizidsubstraten nach 10tägiger Kulturdauer die Pilze immer wieder auf das entsprechende Agarsubstrat übertragen wurden.

Im zweiten Versuchsabschnitt überprüften wir bei den Pilz-Fungizidkombinationen, bei denen sich nach dem Durchlaufen der 8. Passage auf dem Fungizidsubstrat ein signifikant geringerer Hemmungsgrad als bei der 1. Passage zeigte, die Beständigkeit dieser Fungizidtoleranz. Dies geschah durch eine anschließende ein- bis dreimalige 10tägige Kultur der Pilzform auf fungizidfreiem Substrat und erneuter Testung gegenüber dem jeweiligen Fungizid. Der sich dabei ergebende Hemmungsgrad des Pilzes wurde wieder mit dem nach einer erstmaligen Einwirkung des Fungizides verglichen.

Im dritten Versuchsabschnitt gingen wir der Frage nach, inwieweit die als tolerant gegenüber einem Protektiv- oder Systemfunzigid zu bezeichnenden Formen der Pilze zugleich auch widerstandsfähiger gegenüber einem anderen Fungizid sind (Kreuztoleranz). Dazu wurden die gegenüber einem Fungizid toleranten Formen auf Agarkulturen mit wirksamen Dosierungen aller anderen verwendeten Fungizide getestet und auf signifikante Differenzen im Hemmungsgrad zum Verhalten der Normalform des Pilzes bonitiert.

Im vierten und letzten Versuchsabschnitt führten wir jeweils 4fach wiederholte Infektions- und Bekämpfungsversuche mit den signifikant fungizidtoleranten und normalen Pilzformen im Pflanzentest (mit 40 Pflanzen bzw. Blätter) durch. Im Falle von *Fusarium solani f. pisi* wurden die unter Paraffinöl konservierten Kulturen auf 2%iger Biomalznährlösung bzw. in einer Maismehlquarzsandmischung (3 Gew.% Maismehl) vermehrt und zur einheitlichen Verseuchung eines sterilisierten Versuchsbodens (800 mg Pilzmyzel/1 kg Boden bzw. 6 Gewichtsprozent

pilzdurchwachsener Maismehlquarzsand als Zugabe) in Neubauerschalen verwendet. Nach der Verseuchung wurde dem Boden das entsprechende Fungizid in der einschlägigen Dosierung beigemischt und anschließend erfolgte eine Aussaat oberflächendesinfizierter, vorgekeimter Erbsen (*Pisum sativum* L.) oder Bohnen (*Phaseolus vulgaris* L.). Parallel liefen jeweils Varianten ohne Fungizidzusatz bzw. bei den Systemfungiziden Varianten, in denen die Fungizidzugabe nur als vorhergehende Samenbehandlung[1] der Testpflanzen vorgenommen wurde. Zur Kultur der Pflanzen herrschten einheitliche Bedingungen vor. Die Auswertung setzte 8—12 Tage nach der Aussaat ein, indem wir den %-Anteil wurzelkranker durch *F. solani f. pisi* befallener Testpflanzen ermittelten.

Im Falle von *Sclerotinia sclerotiorum* wurde der Laborblattest nach SCHMIDT (1969) verwendet. In großen Petrischalen legten wir auf vorher sterilisiertem Sand-Torfmull-Substrat (7 : 3), das einheitlich mit den zu testenden Pilzformen verseucht war (400 mg Pilzmyzel/1 kg Substrat oder 12% pilzdurchwachsenes Maismehlquarzsandgemisch bezogen auf das Substratgewicht) und dem anschließend die jeweiligen Fungizide in entsprechenden Dosierungen beigemischt waren, sorgfältig abgetrennte Blätter von Salat (*Lactuca sativa* L.) oder Sonnenblumen (*Helianthus annuus* L.) auf. Im Vergleich zu fungizidfreien Kontrollen stellten wir nach einigen Tagen den %-Anteil der von *Sclerotinia* befallenen Blätter fest. In einer anderen Variante zur Pathogenitätstestung wurden abgetrennte Salat- und Sonnenblumenblätter ohne Fungizidzusatz auf steriles, feuchtes Filterpapier ausgelegt und je Blatt mit 3 . . . 4 Myzel-durchwachsenen Agarimpfstücken (7 mm Durchmesser) versehen. Die Befallsermittlung erfolgte auch hier durch Feststellung des %-Anteiles infizierter Blätter. Eine dritte Variante bestand schließlich darin, daß wir bei den Systemfungiziden Blätter von Salat- oder Sonnenblumenpflanzen zur Testung verwendeten, nachdem diese 14 Tage vorher einer Saatgutbehandlung mit den Systemfungiziden unterzogen wurden ('Plantvax' in Naßbehandlung, alle übrigen in Trockenbehandlung). Die das Systemfungizid in mehr oder minder starkem Maße enthaltenden Blätter wurden dann durch Direktbelegen mit Pilz-Impfscheiben oder auf verseuchtem Sand-Torfmull-Substrat hinsichtlich des Befalles untersucht.

Ergebnisse

Die Abbildungen 1 und 2 geben für *S. sclerotiorum*, *R. solani*, *F. solani* f. *pisi* und *B. cinerea* die im Verlaufe von 8 Wuchspassagen auf Agar mit den Protektivfungiziden Zineb, TMTD, Captan, TCDNB (Trichlordinitrobenzol) und PCNB eingetretenen Veränderungen in der Fungizidempfindlichkeit, ausgedrückt im Wirkungsgrad der Fungizide, wieder. Man erkennt zunächst, daß bei den einzelnen Passagen je nach Pilzart, Fungizid und angewandter Dosis beträchtliche

[1] Samenbehandlung bei 'Plantvax' durch 1 h Tauchen in angesetzter Brühe, bei allen übrigen Systemfungiziden trockene Saatgutbehandlung.

Schwankungen im Wirkungsgrad eintraten, die im wesentlichen auf die Variabilität und Heterogenität der Pilzformen zurückgeführt werden. Ungeachtet dieser Schwankungen zeigen sich jedoch beim Vergleich des Wirkungsgrades der Fungizide zwischen der 1. und 8. Passage in Abhängigkeit von Pilzart und Wirkstoff 3 Grundtendenzen, indem entweder der Wirkungsgrad des Fungizides sich nicht wesentlich veränderte, zunahm oder abnahm. Bei *R. solani* (Abb. 1) trat gegenüber allen getesteten Fungiziden während der Passagen keine nennenswerte Änderung ein. Bei *S. sclerotiorum* dagegen bildeten sich bei allen geprüften drei Dosierungen von Zineb, bei geringer Aufwandmenge von Captan und bei den beiden Testungen von PCNB mit signifikanter Differenz zum Ausgangsverhalten nach der 8. Passage gegenüber den genannten Fungiziden weitgehend tolerante Formen heraus. Unter dem Einfluß von TMTD und TCDNB war dieser Effekt nicht, eher noch das Gegenteil, zu beobachten. Auch bei *B. cinerea* (Abb. 2) war auffälligerweise fast durchweg eine Erhöhung der Fungizidempfindlichkeit nach den Passagen festzustellen, in keinem Fall zeigten sich signifikante Toleranzentwicklungen. Bei *F. solani f. pisi* führten wieder mittlere Dosierungen von TMTD, TCDNB und eine geringe Dosierung von Captan nach der 8. Passage zu einer deutlichen Toleranz, während bei PCNB sich das Verhalten des Pilzes nur wenig veränderte.

In den Abbildungen 3 und 4 ist das entsprechende Verhalten der Pilze in den 8 Passagen – zuzüglich des von *P. debaryanum* – gegenüber den Systemfungiziden DCMO ('Vitavax') und DCMOD ('Plantvax') bzw. Thiabendazol dargestellt. Auch hier sind zunächst erhebliche Schwankungen zu verzeichnen. Bei *P. debaryanum* (Abb. 3) konnte durch Thiabendazol keine Wirkung erzielt werden. Nach 8 Passagen auf DCMO- und DCMOD-Agar veränderte sich das Verhalten des Pilzes nicht entscheidend. Auch bei *B. cinerea* war – ähnlich wie bei den Protektivfungiziden – trotz großer Schwankungen bei Thiabendazol, gegenüber allen 3 Systemfungiziden nach den Passagen keine bleibende Empfindlichkeitsveränderung in Richtung einer Toleranzentwicklung wahrzunehmen. Bei *F. solani f. pisi* traten jedoch einheitlich nach der 8. Passage signifikant tolerantere Formen gegenüber DCMO, DCMOD und Thiabendazol auf. Bei *R. solani* (Abb. 4) war dies nicht der Fall. Vergleichbar zu den Protektivfungiziden ergab sich hier keine bleibende Wirkungsänderung bzw. eher eine Zunahme der Empfindlichkeit. Die 8 Passagen von *S. sclerotiorum* führten hingegen bei DCMO zur deutlichen Herausbildung einer weitgehend toleranten Form, nicht aber bei DCMOD und Thiabendazol.

Die Überprüfung der Beständigkeit der sich nach den Passagen gebildeten Toleranz bei *F. solani f. pisi* gegenüber TMTD, TCDNB (die geringe Toleranz gegenüber Captan wurde nicht weiter verfolgt) und den Systemfungiziden DCMO, DCMOD und Thiabendazol sowie bei *S. sclerotiorum* gegenüber Zineb, Captan, PCNB und dem Systemfungizid DCMO ergab ein unterschiedliches Bild (Abbildungen 5 und 6). Bereits nach einer fungizidfreien Kultur war die Toleranz gegenüber TMTD und TCDNB bei *F. solani f. pisi* verschwunden, während bei *S. sclerotiorum* auch nach 2 . . . 3 fungizidfreien Passagen sich die Toleranz gegenüber Zineb, Captan und PCNB erhielt (Abb. 5). Die Toleranz gegenüber den System-

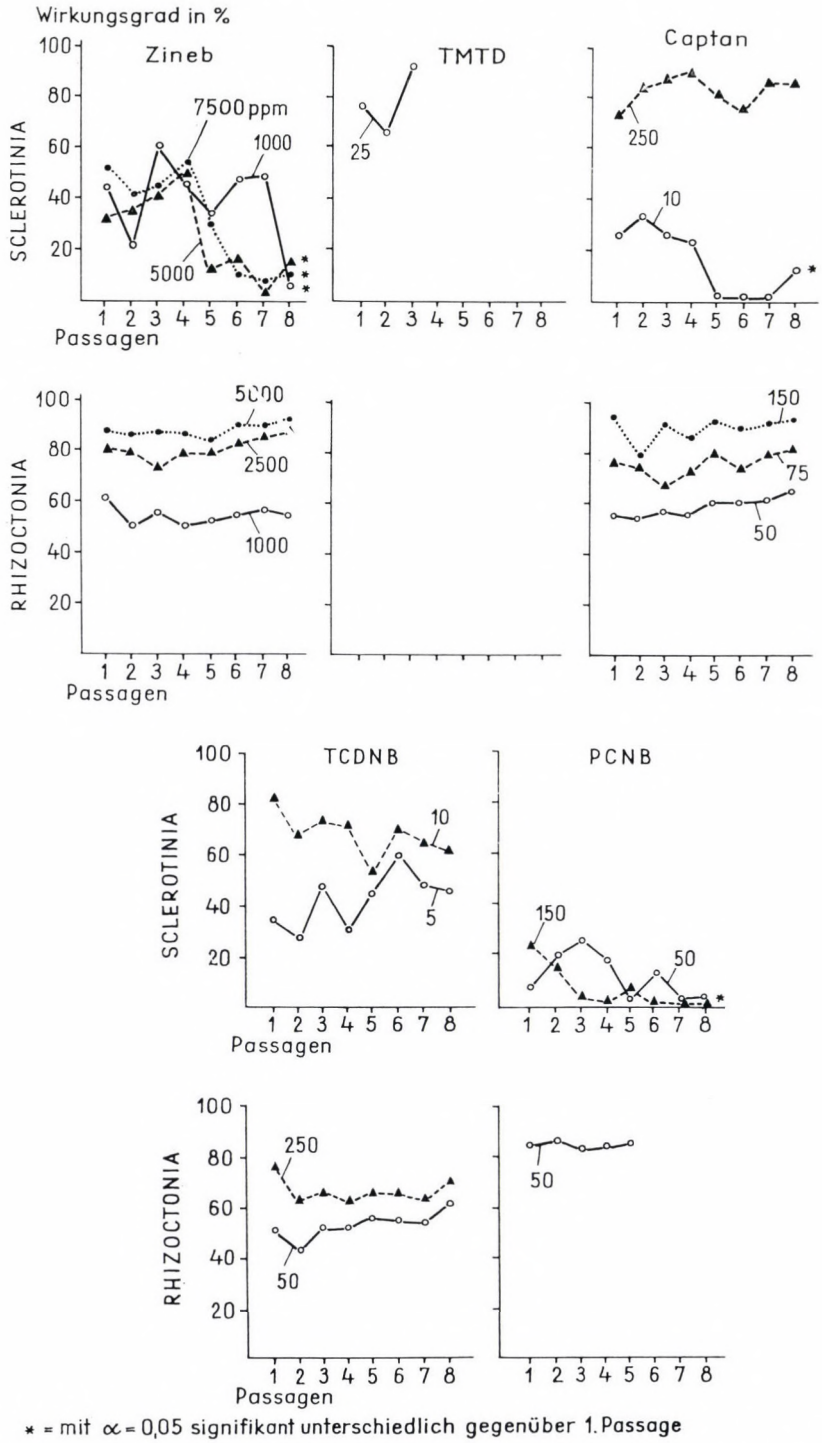

Abb. 1. Veränderungen in der Fungizidempfindlichkeit von *Sclerotinia* und *Rhizoctonia* nach 8maliger Passage auf Agar mit verschiedenen Protektiv-Fungiziden

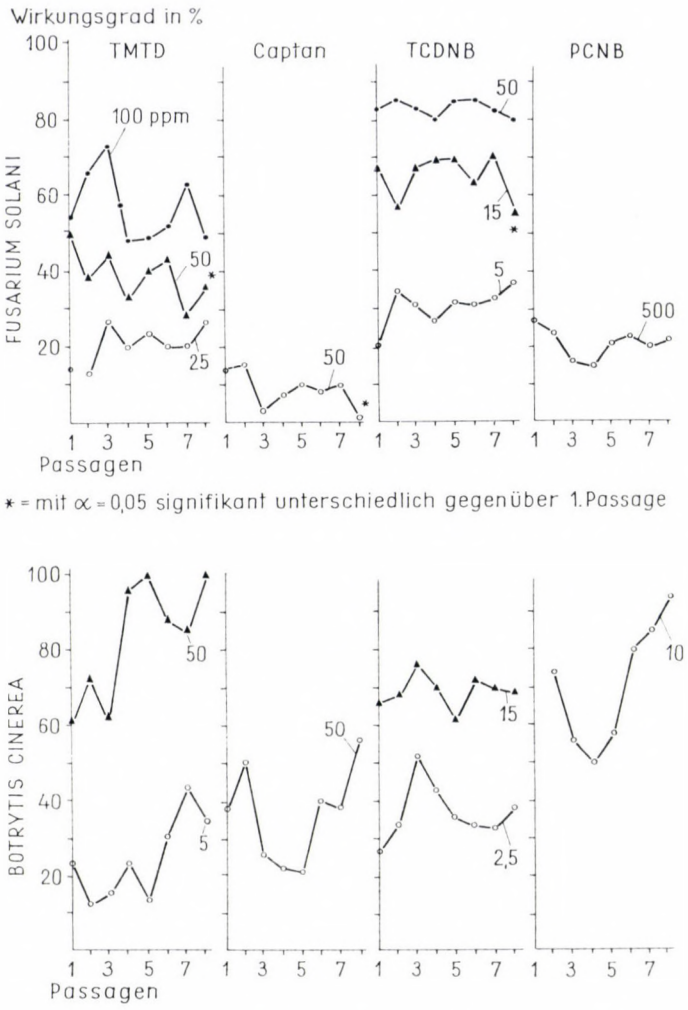

Abb. 2. Veränderungen in der Fungizidempfindlichkeit von *Fusarium* und *Botrytis* nach 8-maliger Passage auf Agar mit verschiedenen Protectiv-Fungiziden

fungiziden erhielt sich bei beiden Pilzen in allen Fällen auch nach 2maliger fungizidfreier Weiterkultur konstant (Abb. 6).

Interessant ist nun, daß die gegenüber den genannten Fungiziden signifikant und konstant toleranten Pilzformen im wechselnder Weise auch kreuztolerant gegenüber anderen System- oder Protektivfungiziden sein können (Abb. 7). Erste

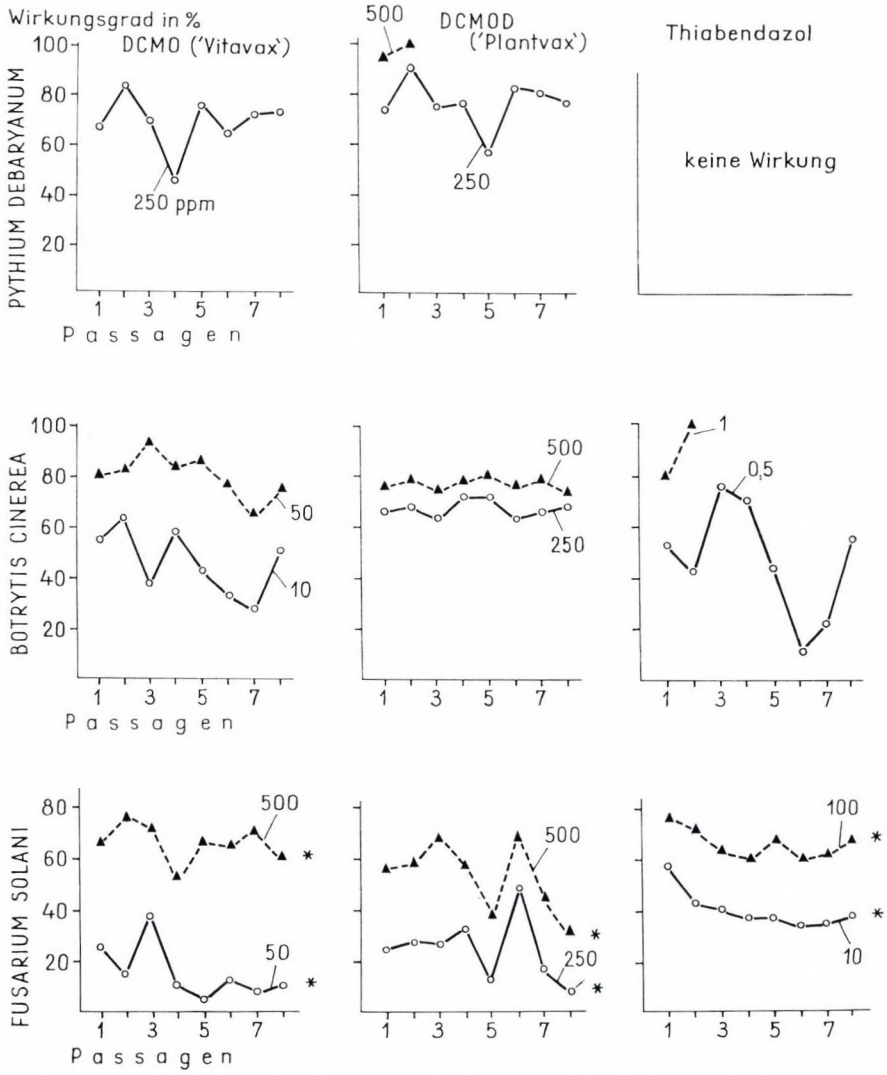

Abb. 3. Veränderungen in der Fungizidempfindlichkeit von *Pythium*, *Botrytis* und *Fusarium* nach 8maliger Passage auf Agar mit verschiedenen Systemfungiziden

Ergebnisse von Kreuztesten weisen dies nach. Allerdings ergeben sich hier offenbar sehr spezifische Verhältnisse, die mit den Besonderheiten des Wirkungsmechanismus der einzelnen Fungizide in Zusammenhang stehen dürften und für die hier noch keine Erklärung gegeben werden kann. So war z. B. die Thiabendazol-tole-

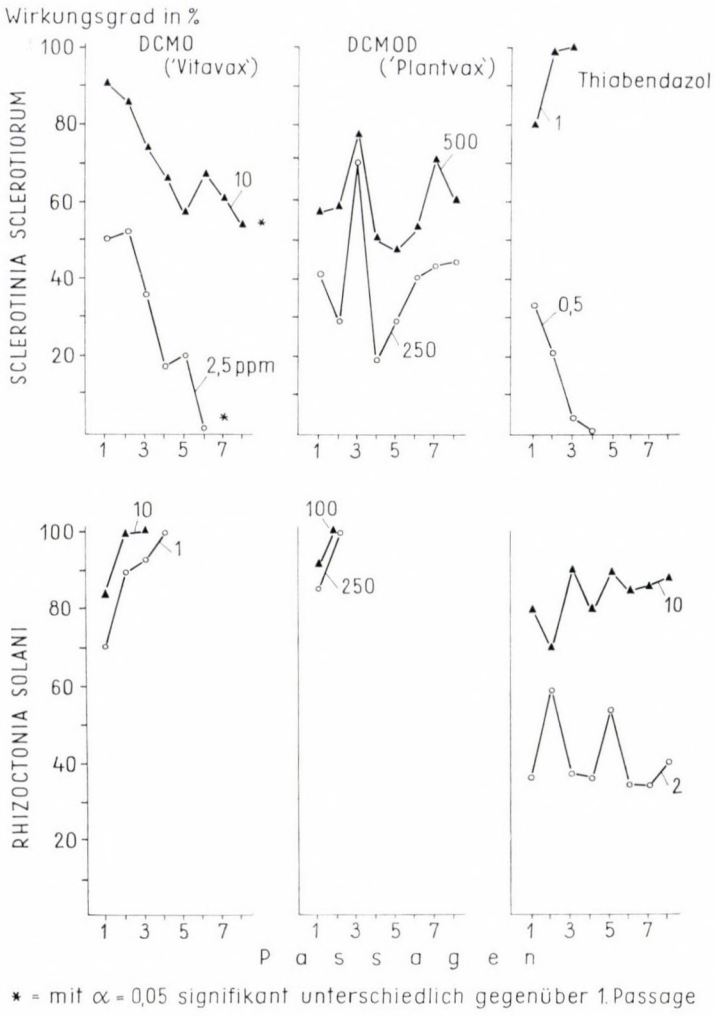

Abb. 4. Veränderungen in der Fungizidempfindlichkeit von *Sclerotinia* und *Rhizoctonia* nach 8maliger Passage auf Agar mit verschiedenen Systemfungiziden

rante *Fusarium*-Form auch tolerant gegen Thiuram, nicht aber umgekehrt. Die DCMO-tolerante Form verhielt sich ebenfalls tolerant gegen DCMOD und Thiabendazol, nicht aber war die DCMOD-tolerante Form auch tolerant gegen Thiabendazol. Auch bei *S. sclerotiorum* waren ähnlich spezifische und noch nicht von allen Seiten durchschaubare Kombinationen zu beobachten. Die Zineb-, Captan- und PCNB-toleranten *Sclerotinia*-Formen waren alle auch signifikant toleranter als der Normaltyp des Pilzes gegen DCMOD. Die DCMO-tolerante

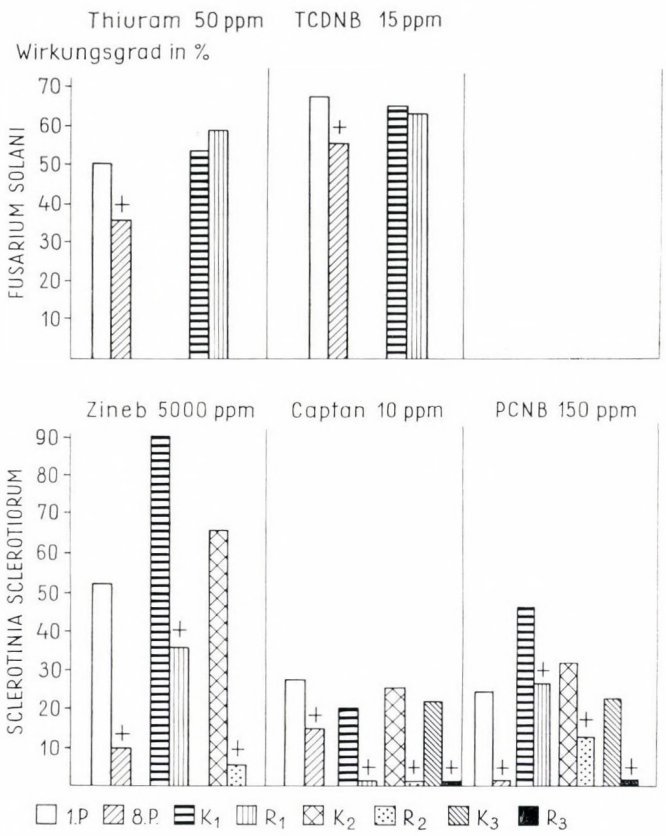

Abb. 5. Beständigkeit der Protektiv-Fungizidtoleranz bei *Fusarium* und *Sclerotinia* nach ein- und dreimaliger Kultur auf fungizidfreiem Agar. 1. P. = 1 Fungizid-Passage; 8. P. = 8 Fungizid-Passage; $K_1 = 8$ Passagen ohne Fungizid, dann 1 Fungizidpassage; $R_1 = 8$ Fungizid-Passagen, eine Passage fungizidfrei, dann wieder Fungizidpassage; $K_2 = 9$ Passagen ohne Fungizid, dann 1 Fungizidpassage; $R_2 = 8$ Fungizid-Passagen, zwei Passagen fungizidfrei, dann wieder Fungizidpassage; $K_3 = 10$ Passagen ohne Fungizid, dann 1 Fungizidpassage; $R_3 = 8$ Fungizid-Passagen, drei Passagen fungizidfrei, dann wieder Fungizidpassage; + = mit $\alpha = 0.05$ signifikant unterschiedlich gegenüber Normalkultur

Form war dagegen zwar tolerant auch gegen DCMOD, bei den Protektivfungiziden aber nur gegen TCDNB. In verschiedenen Fällen scheint jedoch eine kombinierte Toleranz der Pilze gegenüber bestimmten Protektiv- und Systemfungiziden zu bestehen.

Über die Testergebnisse zur Pathogenität und Bekämpfbarkeit der fungizidtoleranten *F. solani f. pisi*-Formen gibt Tabelle 1 Auskunft. An Erbsen war die Pathogenität (ohne Fungizideinsatz) aller fungizidtoleranten Formen nicht unter-

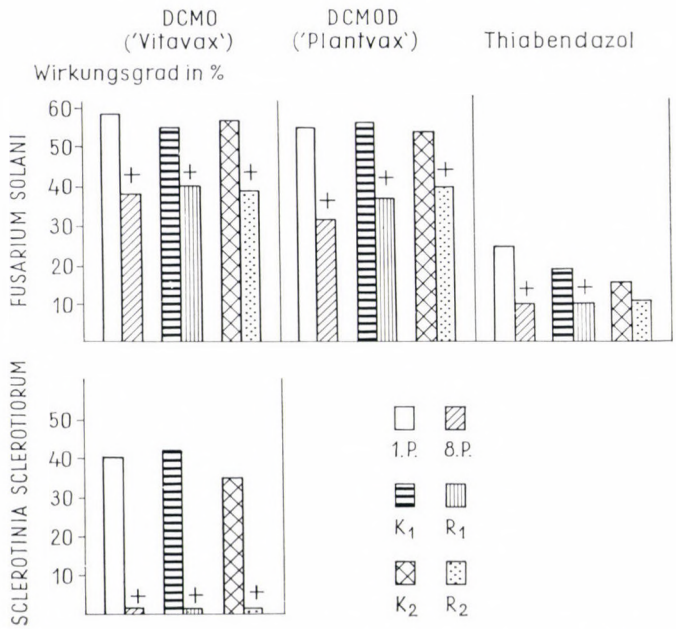

Abb. 6. Beständigkeit der System-Fungizidtoleranz bei *Fusarium* und *Sclerotinia* nach ein- und zweimaliger Kultur auf fungizidfreiem Agar. 1. P. = 1 Fungizid-Passage; 8. P. = 8 Fungizid-Passage; $K_1 = 8$ Passagen ohne Fungizid, dann 1 Fungizidpassage; $R_1 = 8$ Fungizid-Passagen, eine Passage fungizidfrei, dann wieder Fungizidpassage; $K_2 = 9$ Passagen ohne Fungizid, dann 1 Fungizidpassage; $R_2 = 8$ Fungizid-Passagen, zwei Passagen fungizidfrei, dann wieder Fungizidpassage; + = mit $\alpha = 0.05$ signifikant unterschiedlich gegenüber Normalkultur

schiedlich gegenüber der Normalform von *F. solani f. pisi*. An Bohnen lag dagegen die Thiabendazol-tolerante Form in der Pathogenität unter dem Normaltyp. Hinsichtlich der Bekämpfbarkeit war bei der Anwendung der Protektiv- und Systemfungizide zum Boden eine signifikant schlechtere Unterdrückung der TMTD-toleranten Form bei Erbsen mit dem gleichen Fungizid zu verzeichnen. Besonders ausgeprägt hob sich jedoch die praktische Nichtbekämpfbarkeit der Thiabendazol-toleranten *Fusarium*-Form durch entsprechende Applikationen dieses Präparates ab, was sowohl bei Erbsen als auch bei Bohnen erkennbar war. Bei Anwendung dieses Systemfungizides zur Samenbehandlung ergab sich der genannte Effekt nur andeutungsweise. In allen anderen Fällen der Infektions- und Bekämpfungsversuche mit den fungizidtoleranten Formen lagen keine signifikanten Unterschiede zu dem Normaltyp des *Fusarium*-Pilzes vor, da dieser durch die angewandten Fungiziddosen ebenfalls nicht nennenswert unterdrückt wurde.

Bei den Tests mit *S. sclerotiorum* zeigten sich zunächst bei den protektivfungizid-toleranten Formen auffallende Pathogenitätsänderungen gegenüber dem Nor-

		Protektivfungizide				Systemfungizide		
	Zineb	TMTD	Captan	TCDNB	PCNB	DCMO	DCMOD	Thiabendazol
Fusarium	—	⊕	—	—	—	—	—	+
	—	—	—	—	—	+	⊕	⊕
	—	[+]	—	—	—	—	—	—
	⊕	—	—	[+]	⊕	—	—	—
	—	—	—	—	—	⊕	+	—
Sclerotinia	+	—	—	—	⊕	—	⊕	—
	—	—	+	—	⊕	—	⊕	—
	—	—	—	—	+	—	⊕	—
	—	—	—	⊕	—	+	⊕	—

\+ = mit $\alpha = 0{,}05$ signifikant fungizidtoleranter als Normalkultur
— = gleiches Verhalten wie Normalkultur
[ ] = Fungizidtoleranz nicht konstant
⊕ = zusätzlich Fungizidtoleranz neben +

Abb. 7. Das Verhalten bestimmter fungizidtoleranter Formen von *Fusarium solani* und *Sclerotinia sclerotiorum* gegenüber anderen Protektivfungiziden und Systemfungiziden in Agarkultur (Kreuztoleranz)

Tabelle 1

Vergleich der Pathogenität normaler und fungizidtoleranter Formen von *Fusarium solani* an Erbsen und Bohnen. Prozent befallene Pflanzen

1	Fungizidfreier Boden				Fungizidhaltiger Boden wie in Spalte 1 angegeben				Saatgutbehandlung entsprechend wie in Spalte 1 angegeben			
	Erbsen		Bohnen		Erbsen		Bohnen		Erbsen		Bohnen	
	N.	R.	N.	R.	N.	R.	N.	R.	N.	R.	N.	R.
Thiuram 50 ppm	100	100	82	80	71	97	45	47	—	—	—	—
TCDNB 15 ppm	100	100	82	73	100	100	66	65	—	—	—	—
DCMO 50 ppm	100	100	82	50	100	97	68	50	100	97	65	61
DCMOD 500 ppm	58	51	—	—	58	63	—	—	86	85	—	—
Thiabendazol 10 ppm	100	94	82	60	—	—	55	64	—	—	58	43
Thiabendazol 100 ppm	—	—	—	—	1	95	3	50	77	87	28	49

N. = Kontrolle, normale Herkunft
R. = gegenüber angegebenem Fungizid tolerant
— = nicht bestimmt
—— = mit $\alpha = 0.05$ signifikant unterschiedlich

maltyp. Die Normalform ist gegenüber Salatblättern stark pathogen, bei den Zineb-, Captan- und PCNB-toleranten Formen war diese Eigenschaft dagegen zu vermissen. Die gegen das Systemfungizid DCMO tolerante *Sclerotinia*-Form war wie der Normaltyp auch gegen Salat hoch pathogen, zugleich aber auch gegenüber Sonnenblumenblätter, die von dem Normaltyp des Pilzes kaum angegriffen wurden. Hinsichtlich des Bekämpfungseffektes ließ sich feststellen, daß bei den Zineb- und Captan-toleranten *Sclerotinia*-Formen der Einsatz des jeweiligen Fungizides zum Boden – im Vergleich zu fungizidfrei – fast zu einer gewissen Förderung des getesteten Befalles führte, während der Normaltyp des Pilzes mit Ausnahme der angewandten Captan-Dosis unterdrückt wurde. Bei der DCMO-toleranten *Sclerotinia*-Form ergab sich einheitlich, daß diese im Vergleich zum Normaltyp schwieriger mit »Vitavax« zu bekämpfen war. Dieser Befund lag allerdings nur schwach vor, bei Zugabe des Fungizides zum Boden oder bei Verwendung von Blättern der Saatgut-behandelten Testpflanzen mit direkter Beimpfung. Sehr deutlich zeigte er sich aber bei Auslage der Blätter von Saatgut-behandeltem Salat und von Sonnenblumen auf verseuchtem Boden.

Schlußfolgerungen

Die Untersuchungsergebnisse lassen erkennen, daß es bei bestimmten Pilzarten – in unserem Falle *Fusarium solani f. pisi* und *Sclerotinia sclerotiorum* – unter Kulturbedingungen bei einseitigem Fungizidangebot relativ schnell zu einer Selektion fungizidtoleranter Formen kommen kann. Für *F. solani f. pisi* war dabei dieser Vorgang gegenüber den geprüften Systemfungiziden ausgeprägter als gegenüber den getesteten Protektivfungiziden, während bei *Sclerotinia* ein derartiger Unterschied sich nicht bemerken ließ. Sämtliche gegenüber den Systemfungiziden entwickelten Toleranzen blieben aber erhalten auch nach Weiterkultur der Pilzform auf fungizidfreiem Substrat. Toleranzen gegenüber den Protektivfungiziden dagegen verloren sich schnell bei dem *Fusarium*-Pilz und waren nur deutlich konstant bei *Sclerotinia sclerotiorum*. Die systemfungizid- wie auch protektivfungizidtoleranten Formen der Pilze erwiesen sich schließlich nicht weniger pathogen als der normale Ausgangstyp, obgleich sich Verschiebungen im spezifischen Verhalten als Pflanzenkrankheitserreger ergaben. Auch eine schwierigere Bekämpfbarkeit der toleranten Formen im natürlichen Infektionsversuch bestätigte das Verhalten der Erreger im Labor, wobei dies auch für die Systemfungizide besonders deutlich war. Berücksichtigt man weiterhin, daß es Fälle gab, wo sich ein tolerantes Verhalten gegenüber einem Systemfungizid mit einer gleichfalls verminderten Empfindlichkeit gegenüber einem Protektivfungizid und umgekehrt kombinierten, so liegen Hinweise für die Notwendigkeit vor, beim Einsatz von Systemfungiziden in stärkerem Maße an Möglichkeiten einer pilzlichen Toleranzentwicklung zu denken. Eine Übertragung aller diesbezüglich von den klassischen Fungiziden herrührenden Vorstellungen scheint deshalb nicht in jedem Falle richtig.

Tabelle 2

Vergleich der Pathogenität normaler und fungizidtoleranter Formen von *Sclerotinia*

1	Der Pilz wurde direkt auf die Blätter aufgetragen							
	Fungizidfrei				Blätter von Pflanzen mit Saatgutbehandlung wie in Spalte 1 angegeben			
	Salat		Sonnenbl.		Salat		Sonnenbl.	
	N.	R.	N.	R.	N.	R.	N.	R.
Zineb 5000 ppm	57	0	—	—	—	—	—	—
Captan 10 ppm	57	5	—	—	—	—	—	—
PCNB 150 ppm	57	21	—	—	—	—	—	—
DCMO 10 ppm	100	100	15	100	70	100	12	100

N. = Kontrolle, normale Herkunft
R. = gegenüber angegebenem Fungizid tolerant
— = nicht bestimmt
—— = mit $\alpha = 0.05$ signifikant unterschiedlich

Zusammenfassung

5 phytopathogene Pilzarten *(Pythium debaryanum, Sclerotinia sclerotiorum, Botrytis cinerea, Fusarium solani* f. *pisi, Rhizoctonia solani)* wurden in wiederholten Passagen auf Agar mit Zusätzen von Protektivfungiziden (Dithiocarbamaten, TMTD, Captan, Chlornitrobenzole) und Systemfungiziden (Oxathiine, Thiabendazol) kultiviert. Aus der Ausgangsform von *Fusarium solani* f. *pisi* gelang es im Vergleich zu den Protektivfungiziden leichter, gegenüber Systemfungiziden tolerante Formen zu selektieren. Bei *Sclerotinia sclerotiorum* traten tolerante Formen sowohl gegenüber einem Systemfungizid als auch gegenüber Zineb, Captan und PCNB auf.

Für *Rhizoctonia solani*, *Botrytis cinerea* und *Pythium debaryanum* waren Toleranzbildungen nicht festzustellen. Die selektierten fungizid-toleranten Pilzformen behielten diese Eigenschaft auch bei nachfolgender Kultur unter fungizidfreien Bedingungen bei. Die Toleranz der Pilzformen gegenüber einem Fungizid war sowohl bei System- als auch bei Protektivfungiziden in spezifischer Weise mit Toleranzerscheinungen auch gegenüber anderen Wirkstoffen verbunden (Kreuztoleranz). Pathogenitätsprüfungen ergaben, daß auch in dieser Hinsicht bei den fungizid-toleranten Formen Veränderungen eintreten, obgleich nicht in Richtung einer generellen Pathogenitätsverminderung. In Befallsversuchen waren die pathogenen systemfungizidtoleranten Pilzformen durch die betreffenden Fungizide nicht mehr normal bekämpfbar. Die Bedeutung der Toleranzbildung gegenüber Systemfungiziden wird abschließend herausgestellt.

sclerotiorum an Salat- und Sonnenblumenblättern. Prozent befallene Blätter im Blattest

Der Pilze wurde dem Boden beigemischt											
Fungizidrei				Boden + Fungizid wie in Spalte 1 angegeben				Blätter von Pflanzen mit Saatgutbehandlung wie in Spalte 1 angegeben			
Salat		Sonnenbl.		Salat		Sonnenbl.		Salat		Sonnenbl.	
N.	R.	N.	R.	N.	R.	N.	R.	N.	R.	N.	R.
70	0	—	—	0	12.5	—	—	—	—	—	—
70	0	—	—	70	7.5	—	—	—	—	—	—
70	0	—	—	2.5	0	—	—	—	—	—	—
15	100	20	83	10	100	17	80	0	65	0	60

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Begrenzende Faktoren für die Aufnahme von Streptomycin in Hopfen*

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The systemic action of antibiotica inhibiting *Pseudoperonospora humili* in hops as exemplified in streptomycin was demonstrated. The uptake, transport and deposition of tritium-labelled streptomycin have been investigated in model experiments. No transport or uptake of streptomycin occurred in the case of leaves. On the contrary, hop plants having a root system easily absorb and translocate tritium-labelled streptomycin, which accumulates mainly in the leaves. Young shoots were also active in uptake and transport of the antibioticum through the internodia. The internodia of old shoots were unable to transport streptomycin.

Zu den schwer bekämpfbaren Pilzen zählt der Erreger des falschen Mehltaus des Hopfens, *Pseudoperonospora humuli* (Miy. et Tak.) Wils. (Abb. 1). Die systemisch infizierte Pflanze ist mit herkömmlichen Mitteln nicht zu heilen, Sekundärinfektionen sind nur durch hohen Spritzaufwand zu verhindern. Neue Möglichkeiten der Bekämpfung des Pilzes eröffneten die Befunde von HORNER und MAIER (1957) sowie MAIER und HORNER (1957) durch den Einsatz des Streptomycin. Die systemische Wirkung des Antibiotikums wurde in der Folgezeit mehrfach bestätigt, so u. a. von COLEY-SMITH (1963, 1966), HORNER (1963), MAIER (1959, 1960) und SCHICKE (1961). Das Antibiotikum hemmt die Zoosporangienkeimung (ACIMOVIC und MIJAVEC (1964) und -produktion (Horner, 1963). Diese Inhibitorwirkung des Streptomycins auf Wachstum und Entwicklung wurde auch für andere *Peronosporaceen* beschrieben, so u. a. von VÖRÖS (1967).

Die Befunde über die Geschwindigkeit der Aufnahme und die Wirkung des Antibiotikums sind unterschiedlich. In Lanolinpaste formuliertes Streptomycin wird in Nitratform schneller als Streptomycinsulfat aufgenommen (MAIER und HORNER, 1957). Meist wurden junge Pflanzen mit dem Antibiotikum gespritzt. Die Ergebnisse der Streptomycinbehandlungen waren jedoch unterschiedlich. Während ACIMOVIC und MIJAVEC (1964) in Gewächshausversuchen keine befriedigende Wirkung erzielen konnten, wird von anderen Autoren über die erfolgreiche Bekämpfung von *Pseudoperonospora humuli* mit Streptomycin berichtet. SCHICKE

* Herrn Professor Dr. A. HEY zum 65. Geburtstag gewidmet.

Abb. 1. Gestauchte Triebspitze an Hopfen — sog. »Bubikopf« nach Befall mit *Pseudoperonospora humuli*

(1961) erreichte durch zweimalige Behandlung junger Hopfentriebe mit 1000 ppm Streptomycin neben einem kurativen Effekt für 6 Wochen ausreichenden Schutz gegen Sekundärinfektionen.

Wir stellten in unseren Arbeiten fest, daß die Behandlung von Pflanzen unterschiedlichen Alters zu widersprüchlichen Ergebnissen führt, die ein Urteil über die optimale Applikationsform des Antibiotikums ausschließt. Vorversuche an Pflanzen gleichen Alters ergaben, daß ferner Streptomycin nicht immer systemisch wirkt. Unsere Untersuchungen dienten daher dem Ziel, Ursachen zu erkennen, die einer Aufnahme des Antibiotikums und damit seiner systemischen Wirkung entgegenstehen.

In vergleichenden Untersuchungen setzten wir erstmals neben Streptomycinsulfat auch Tritium-markiertes Streptomycin ein. Es wurde durch Wilzbach-Reaktion tritiert und über Ionenaustauscher gereinigt. Dabei entstanden ^{3}H-Dihydro-Streptomycin und uniform markiertes Streptomycin, wobei der Anteil

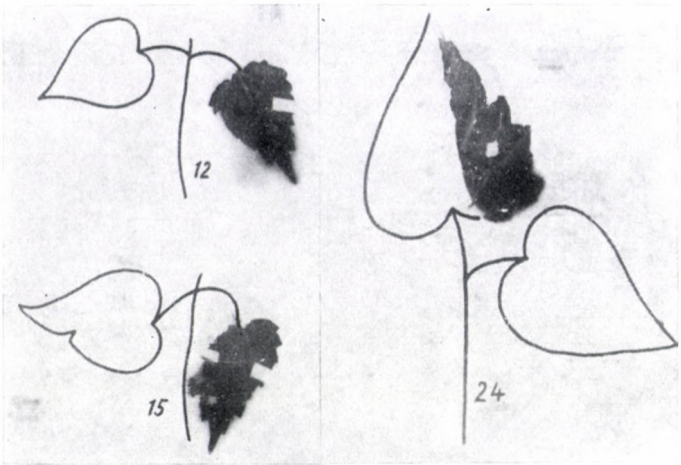

Abb. 2. Autoradiogramme dekaptierter, beblätterter Sproßabschnitte nach Blattapplikation. Links: Blatt-Blatt-Transport; rechts: Halbseitige Blattapplikation

des ^{3}H-Dihydro-Streptomycin größer war. Über die Herstellung dieser Substanz wurde bereits an anderer Stelle berichtet (EISENBRANDT, 1970).

Die Untersuchungen zeigten, daß das Antibiotikum nur über bestimmte Sproßabschnitte in die Pflanze gelangt und daß das Alter der Pflanze für die Aufnahme des Wirkstoffs Grenzen setzt.

So wird Streptomycin nicht vom *Blatt* aufgenommen. In unbehandelten Pflanzenabschnitten konnten wir nach Blattapplikation kein Streptomycin nachweisen. Sowohl ein- als auch dreimalige Spritzungen mit 500 und 1000 ppm-haltigen Wirkstofflösungen blieben ohne Erfolg. Dieser Befund wurde auch durch die Ergebnisse mit ^{3}H-Streptomycin durch Ermitteln der Impulsrate von Blattpreßsäften bestätigt. ^{3}H-Streptomycin wird auch nicht in Sproßabschnitten von Blatt zu Blatt und von Blatthälfte zu Blatthälfte transportiert (Abb. 2). Es besteht kein Unterschied zwischen jungen und alten Blättern. Der Einsatz von DMSO (0.1 und 1%) förderte die Blattaufnahme nicht.

Bewurzelte Hopfenpflanzen nehmen dagegen ^{3}H-Streptomycin auf (Abb. 3). Es wird nach autoradiographischen Befunden in alle Pflanzenteile transportiert. Starke Anreicherungen fanden wir in den Blattadern und an den Blatträndern. Zusätzlich zum autoradiographischen Nachweis wurde die Aktivität der Substanz durch Zählen der Impulsrate in Pflanzenpreßsäften ermittelt. Nach Wurzelapplikation lagen die Impulsraten bereits nach 3 Stunden über denen der unbehandelten Kontrolle. Es wurde die Aufnahme des Antibiotikums nach 3, 6 und 12 Stunden sowie nach 1, 3, 5 und 10 Tagen verfolgt. Wie in entsprechenden Gewächshausversuchen mit nichtmarkiertem Wirkstoff nahm der Antibiotikumgehalt in den Sprossen nach oben hin ab, in den Blättern apikaler Bereiche jedoch zu (Abb. 4).

Abb. 3. Autoradiogramm einer Hopfenpflanze nach Sproßaufnahme von [^{3}H]-Streptomycin (Applikationslösung: 1000 ppm Streptomycinsulfat mit einem [^{3}H]-Streptomycinsulfatgehalt von 16.6 μCi/ml; Applikationszeit: 3 Tage; Expositionszeit: 25 Tage)

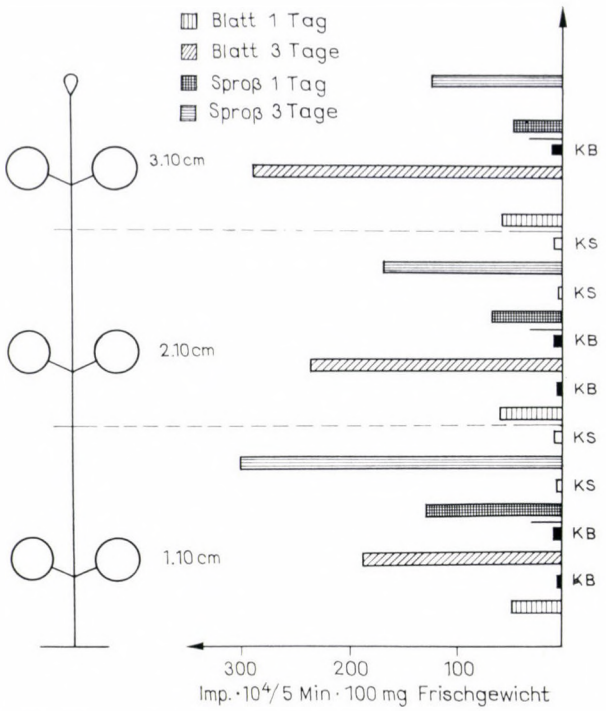

Abb. 4. Transport von [^{3}H]-Streptomycin im Hopfen, 1 und 3 Tage nach Wurzelapplikation (Applikationslösung: 1000 ppm Streptomycinsulfat mit einem [^{3}H]-Streptomycinsulfatgehalt von 3.5 μCi/ml)

Wir konnten nach Wurzelapplikation noch nach 41 Tagen die antibiotische Substanz in Sproß und Blättern von Fechserpflanzen nachweisen. Dies war jedoch nicht mehr zur Zeit der Ernte im Erntegut möglich.

Da bei Spritzapplikation die Aufnahme des Streptomycins durch das Blatt auszuschließen ist, wurde die Aufnahme des Wirkstoffs über die *Internodien* junger Pflanzen eingehend untersucht. Wir behandelten gezielt Internodien von Pflanzen mit einer durchschnittlichen Höhe von ca. 40 cm. Spätestens nach 24 Stunden konnten wir das Antibiotikum im Bereich der Sproßspitze nachweisen. Es wird in erster Linie apikal transportiert und aus dem Sproß in die Blätter weitergeleitet. Der Abwärtstransport des Wirkstoffs ist frühestens nach dem dritten Tag nachweisbar, so daß er noch nicht als gesichert angesehen werden kann (Abb. 5). Im Gegensatz zur Aufnahme des Wirkstoffs über die Internodien junger Pflanzen wird das Antibiotikum auch im Bereich der Internodien alter verholzter Pflanzen nicht aufgenommen. Wir konnten das in Freilandversuchen nachweisen.

Fassen wir diese Befunde zusammen, so bleibt die Feststellung, daß der systemischen Wirkung des Streptomycins Grenzen gesetzt sind. Das Aufnahmever-

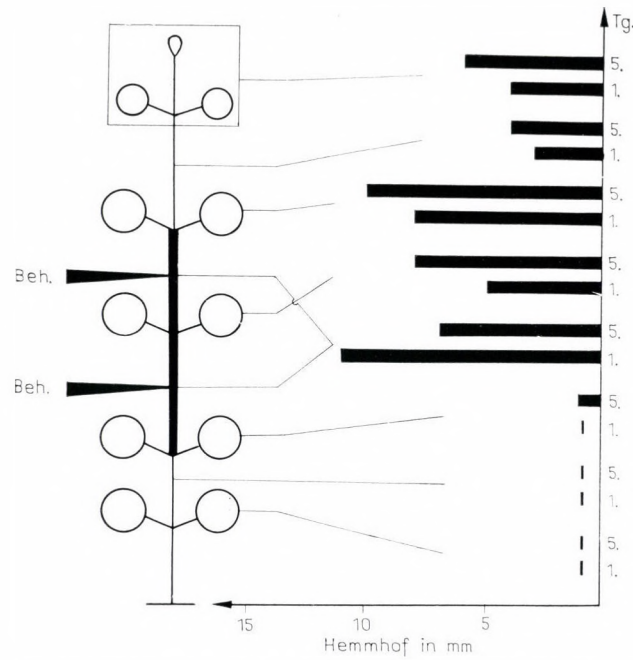

Abb. 5. Aufnahme von Streptomycin nach Sproßapplikation in Hopfen und die Verteilung in der Pflanze

mögen wird durch das pflanzliche Organ und das Alter der Pflanze maßgeblich bestimmt. Somit hat die Aufnahme des Antibiotikums unmittelbare Beziehung zum Transport des Streptomycins im Hopfen (Abb. 6): Streptomycin wirkt nach Wurzel- und Blattapplikation systemisch. Der Wirkstoff wird nicht vom Blatt aufgenommen. Der praktische Einsatz des Antibiotikums bei Spritzungen ist nur dann sinnvoll, wenn der Sproß ausreichend getroffen wird und nur, wenn junge Pflanzen, deren Gewebe wenig verhärtet ist, behandelt werden. Spätspritzungen zur Behandlung der Zapfeninfektion zeigten keinen systemischen Effekt.

Zusammenfassung

Am Beispiel des Streptomycins wird die systemische Wirkung des gegen *Pseudoperonospora humuli* wirkenden Antibiotikums im Hopfen demonstriert. Durch vergleichende Untersuchungen mit nichtmarkiertem und markiertem Antibiotikum werden Modelluntersuchungen über Aufnahme, Transport und Speicherung des Streptomycins dargestellt. Streptomycin wurde nicht vom Blatt aufge-

Tabelle 1

Saatgutbeizungsversuche mit systemischen Fungiziden (Vitavax, Plantvax und JF 2305) gegen Kopfbrand. Die Prozentwerte der erkrankten Pflanzen. Gábormajor—Agárd, 1969

Linien	Versuchsstelle	V_1	V_3	P_1	P_3	JF_1	JF_3	K
014	Gábormajor	70	75	55	55	57	39	59
014	Agárd	68	64	63	54	64	71	62
156	Gábormajor	19	26	13	28	11	17	14
156	Agárd	7	16	10	14	18	15	14
C 103	Gábormajor	5	16	20	5	17	14	6
C 103	Agárd	10	5	14	12	9	12	15
WF 9	Gábormajor	12	0	4	4	5	0	0
WF 9	Agárd	3	4	4	2	2	0	4

V = Vitavax
P = Plantvax
K = Kontrolle
1 = Minimaldose
3 = Dreifache Dose

Tabelle 2

Saatgutbeizungsversuche mit systemischen Fungiziden (Vitavax, Plantvax und JF 2305) gegen Kopfbrand. Die Prozentwerte der erkrankten Pflanzen. Gábormajor —Agárd, 1969

Hybriden	Versuchsstelle	V_1	V_3	P_1	P_3	JF_1	JF_3	K
C5×014	Gábormajor	19	32	27	38	44	33	26
C5×014	Agárd	34	32	21	20	27	36	35
Mv 5	Gábormajor	40	28	30	30	21	40	28
Mv 5	Agárd	18	13	19	10	17	24	21
43D×C103	Gábormajor	56	55	53	50	43	54	65
43D×C103	Agárd	32	42	31	39	29	39	35
156×C103	Gábormajor	27	26	27	34	34	23	35
156×C103	Agárd	15	7	11	8	9	13	8

V = Vitavax
P = Plantvax
K = Kontrolle
1 = Minimaldose
3 = Dreifache Dose

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Versuche mit systemischen Fungiziden gegen Kopfbrand des Maises

Von

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Seeds of 4 inbred lines and 4 hybrids of corn have been treated with Vitavax, Plantvax and JF 2305, to control head smut *(Sphacelotheca reiliana)*. Results are presented in Tables 1 and 2.

Der Kampf gegen den Kopfbrand, *Sphacelotheca reiliana* (Khuen) Clint., des Maises in Martonvásár hat im Jahre 1959 begonnen. Die Lösung stand immer vor uns in der Resistenzzüchtung, einerseits weil wir schon in den ersten Jahren entschiedene Differenzen an Feldresistenz beobachteten, anderseits gibt es keine entsprechenden Fungizide bzw. ist ihre Anwendung nicht ökonomisch. Trotz dieser Lage waren diesbezügliche Probleme beständig interessant für uns. Als die ersten systemischen Fungizide erschienen, haben wir einige Zusammenhänge angenommen: Kopfbrand ist eine bodenbürtige Krankheit, die durch ihre Hyphen systemisch in das Gewebe des Maisstengels hervordringt und die generativen Organe beschädigt.

Vor einigen Jahren erhielten wir schon Informationen, daß Oxathiin-Derivate gegen Heterobasidiomyzetiden, darunter Brandkrankheiten bei Gerste, Gräser usw. erfolgreich sind. Aus praktischen und ökonomischen Gründen haben wir i. J. 1969 Saatgutbeizung mit drei systemischen Fungiziden angewandt: Vitavax (2,3-Dihydro-5-Carboxanilido-6-Methyl-1,4-Oxathyin), Plantvax (2,3-Dihydro-5-Carboxanilido-6-Methyl-1,4-Oxathyin-4,4-Dioxid) und ein flüssiges Mittel JF 2305. Als Testpflanzen dienten verschieden kopfbrandanfällige vier Inzuchtlinien und vier Hybriden. Den Vorschriften gemäß wendeten wir die Dosen in minimaler, zweifacher und dreifacher Menge an. Die Versuche wurden in unserem Provokationsgarten für Kopfbrand und auf einem Feld natürlich stark infiziert in Agárd, Komitat Fejér, angebaut in Wiederholungen mit 30 bzw. 50 Pflanzen je Parzelle. Phytotoxische Wirkungen konnten wir nicht beobachten. Die anfälligsten Mais-Formen erkrankten bis zu 70 Prozent ohne konsequente Differenzen zwischen den gebeizten Behandlungen und den Kontrollen.

Die durchschnittlichen Befallswerte der Inzuchtlinien sind in Tabelle 1, die der Hybriden in Tabelle 2 mitgeteilt.

Es könnten diskutiert werden: Infektionsmöglichkeiten des Kopfbrandes

COLEY-SMITH, J. R. (1966): Early-season control of hop downy mildew, Pseudoperonospora humuli (Miy. et Tak.) Wils., with streptomycin and protectant fungicides in severely infected plantings. Ann. appl. Biol., London *57*, 183—191.

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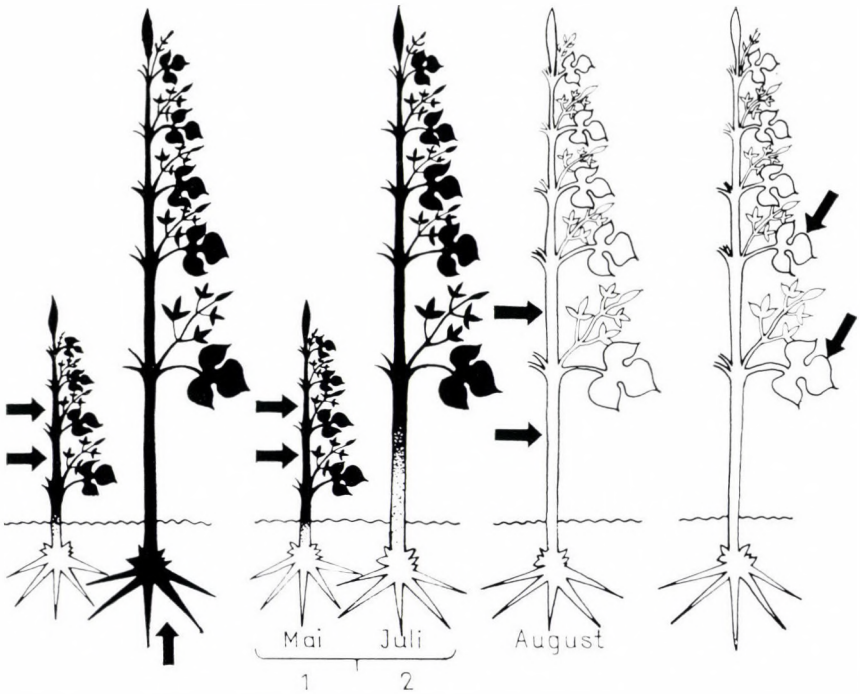

Abb. 6. Aufnahme und Transport (schwarz) von Streptomycin in Hopfen: Links: Wurzelapplikation und Applikation über Internodium (Gewächshausversuch). Mitte 1: Applikation über Internodium und Verteilung in junger Pflanze. Mitte 2: Verteilung von Streptomycin in der gleichen Pflanze nach fortgeschrittenem Wachstum. Mitte rechts: Applikation von Streptomycin über Internodien alter Pflanze. Rechts: Applikation von Streptomycin über Blätter alter Pflanze

nommen. Bewurzelte Hopfenpflanzen nehmen dagegen nach autoradiographischen Befunden Tritium markiertes Streptomycin auf und transportieren es gleichmäßig in alle Pflanzenteile. Das Antibiotikum war besonders stark in den Blattadern und an den Blatträndern angereichert. Bedeutsam ist die Sproßaufnahme des Antibiotikums bei *jungen* Pflanzen.

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Coley-Smith, J. R. (1963): Pathology section, the years work. Wye College, Dep. of Hop Research, Annual Report 1962, Ashford, Kent, 17–19 und 20–21.

bis zum 8–10blättrigen Pflanzenstadium (20–30 cm an Höhe) und Pflanzenmenge; die Menge und Auftragemethoden des Fungizides an die Oberfläche der Maiskörner; die transportablen Fungizide und die Differenzen zwischen den die systemische Infektion hindernden Stoffen; Probleme der Selektivität der systemischen Fungiziden.

Wir sind zweifellos am Anfang der Forschung des Wirkungsmechanismus der systemischen Fungiziden. Es ist vorstellbar, daß der Kopfbrand des Maises ein gutes – wahrscheinlich nicht leichtes – Testobjekt ist. Auf diesem Gebiet wäre eine engere Zusammenarbeit von Pflanzenschutzbiochemikern und Resistenzzüchtern nötig.

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