Spread of *Tomato spotted wilt virus* and population development of *Frankliniella occidentalis* in pepper resistant to thrips

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The effect of thrips resistance on both the spread of *Tomato spotted wilt virus* (TSWV) and the population development of its major vector *Frankliniella occidentalis* was analysed on resistant and susceptible pepper (*Capsicum*) accessions. After release of viruliferous thrips, spread of TSWV was significantly lower in the primary and delayed in the secondary infection phase in plots with the thrips-resistant accession CPRO-1 compared to plots with the thrips susceptible accession Pikante Reuzen. Similar results were obtained in plots with a 1:1 mixture of plants of both accessions. Spread to the virus-resistant accessions PI 152225 and PI 159236, resistant and susceptible to thrips, respectively, was delayed in the secondary phase to plants of the former accession. Since the delay of the infection in the plots with thrips-resistant plants could only partly be explained by an impeded development of thrips populations, other factors, probably related to the behaviour of thrips, also affect the transmission of TSWV to the resistant plants. The results obtained indicate that thrips resistance in pepper can be a useful tool in IPM strategies to control TSWV infections.

Keywords: Frankliniella occidentalis, TSWV, vector resistance, virus resistance, virus transmission

Frankliniella occidentalis Pergande (Thysanoptera; Thripidae) is not only an important pest in many agricultural crops throughout the world, but is also one of the main vectors of *Tomato spotted wilt virus* (TSWV). This virus causes severe diseases in many crops like groundnut, sweet pepper, tobacco, and tomato in the subtropics and in many greenhouse-grown crops and ornamentals in temperate regions. The virus is transmitted in a propagative/circulative manner to a host range of at least 1000 plant species (Chatzivassiliou *et al.*, 2001). Control of TSWV is mainly based on the application of pesticides to reduce the vector population, and alternatively on measures as removal of infected plants, and the use of virus free stock material. Due to increased public awareness of potential hazards to the environment of pesticides, interest in breeding for pest resistance for controlling the spread of pathogens has increased ever since the 1960s (Kennedy, 1976).

Several studies have shown that vector resistance prevents virus spread (Amin, 1985; Rizvi & Raman, 1983; Berlinger *et al.*, 1986; Bouguet, 1981; Parejarearn *et al.*, 1984). However, resistance to insects in crops is not always effective in preventing virus spread and even increases of virus incidence have been reported (Baerecke, 1958; van de Wetering, 1999). The effect of vector resistance on the incidence has to be explained by interactions between vector, virus, and plant, and mechanisms by which the virus is transmitted. However, a detailed analysis on virus spread and vector population dynamics has not been made for most crops with vector resistance. The objective of this study was to analyze the spread of TSWV in thrips-resistant pepper under greenhouse conditions, in relation to thrips population development.

MATERIAL AND METHODS

Pepper accessions, thrips population and virus isolate

The *Capsicum annuum* accessions Pikante Reuzen and CPRO-1, and the *Capsicum chinense* accessions PI 152225 and PI 159236 were used in this study. The accessions CPRO-1, resistant to thrips, and Pikante Reuzen, susceptible to thrips, are equally susceptible to TSWV (Maris *et al.*, 2003). Accessions PI 152225, resistant to thrips, and PI 159236, susceptible to thrips (Maris *et al.*, 2003), were known to be resistant to TSWV (Black *et al.*, 1991; Boiteux & de Ávila, 1994).

The *F. occidentalis* population used was isolated from a greenhouse infestation in lucerne (*Medicago sativa*). To produce viruliferous thrips, 0-4 h old larvae were confined to leaf disks (2.5 cm in diameter, i.d.) from TSWV-infected *Datura stramonium* leaves in a Petri dish (3.4 cm i.d.) on 1.5% agar. After an acquisition access period of 48 h, the larvae were transferred to virus-free leaf disks of *D. stramonium* until the adults emerged. The percentage of viruliferous adults was determined by testing 100 randomly sampled adults on Petunia leaf disks (Wijkamp & Peters, 1993).

The TSWV isolate BR01 (de Ávila *et al.*, 1990) was used in this study.

Spread of TSWV and population development of its vector in thrips-resistant and susceptible pepper

Spread of TSWV in thrips-resistant pepper was studied in a greenhouse (22x17x4 m) experiment. Twenty-five plots with 25 4-weeks-old pepper plants were laid out in a greenhouse. The analysis was done in 20 plots with plants of the pepper accession CPRO-1, Pikante Reuzen, PI 152225 or PI 159236 (non-choice plots), and in 5 plots with a 1:1 mixture of CPRO-1 and Pikante Reuzen plants (choice plots). Treatments were assigned to the plots in a Latin-square design. Plants were grown in 5-l plastic pots and placed at a distance of 50 cm between and 45 cm within the rows. The plots were surrounded by 'edge-plots' to prevent environmental influences affecting the outer-plots. Plants were watered daily with 200 ml water using a dripping-system, and grown in a daily 16-h light and 8-h dark regime. The experiment was conducted from February to April in Wageningen, the Netherlands. The temperature in the greenhouse ranged from 23-31ºC at daytime and was kept at 20ºC during the night.

Forty randomly selected adults that were allowed to acquire TSWV as 0-4 old larvae on infected leaf disks were released in each plot at 5 spots. Plants were monitored for infection by visual inspection and by ELISA. The number of thrips on the plants was counted by visual inspection.

The percentages of virus-infected plants found after each weekly interval was analysed as binomial distributed variables with GENSTAT and compared for each treatment (Payne *et al.*, 1993). The number of thrips found on the plants was analysed by the Kruskal-Wallis one-way analysis of variance method.

Susceptibility of PI 152225 and PI 159236 accessions to TSWV by thrips-mediated inoculation

The inoculation efficiency of viruliferous thrips on the virus-resistant PI 152225 and PI 159236 accessions was studied by exposing 4-weeks-old plants to 1, 2, 4, 8 or 16 viruliferous adult thrips. Each plant was individually caged with a transparent cylinder closed with thrips proof gauze at the top. Thrips could feed on the plants for 7 days, after which they were removed from the plants with an aspirator. During this inoculation period the thrips were daily counted. Seven days after removal of the thrips, the local lesions produced were counted. There were 10 replicates per pepper accession for all treatments. The number of local lesions formed was related to the product of thrips numbers and the number of days that thrips were recovered.

RESULTS

Reduced spread of TSWV and development of thrips in vector-resistant pepper

The spread of TSWV was studied in thrips-resistant pepper by releasing viruliferous thrips in plots with plants of thrips-resistant (CPRO-1 and PI 152225) and susceptible (Pikante Reuzen and PI 159236) accessions. Using a *Petunia* leaf disk assay (Wijkamp & Peters, 1993), 53% of the released adults were capable to transmit TSWV, hence approximately 21 of the 40 specimens, released per plot, were viruliferous. The release of 40 thrips in each plot at five spots resulted in an equal infection pressure over the whole experiment.

The first systemically infected CPRO-1 and Pikante Reuzen plants in the non-choice plots were observed two weeks after thrips release (Fig. 1). After the appearance of the first infected

plants, the virus incidence in CPRO-1 plots progressed at a significant lower rate than in the Pikante Reuzen plots ($P<0.05$). Nevertheless, 12 weeks after the release of the thrips, 97% of the CPRO-1 plants and 100% of the Pikante Reuzen plants were infected. The number of thrips observed was significantly lower on the former than on the latter plants during the whole experiment ($P<0.05$). Thrips could rarely be observed on the CPRO-1 plants in the first 7 weeks after their release, but their number increased rapidly after the appearance of the first flowers. With the increase of thrips on the TR plants, the number of infected plants started to increase more rapidly (Fig. 1).

The first infected Pikante Reuzen and CPRO-1 plants in the plots with a 1:1 mixture of plants were also found two weeks after the release of the thrips (Fig. 2). Virus incidence on CPRO-1 and Pikante Reuzen plants in these plots developed at a similar rate as in the single cultivar plots. Again the thrips numbers on the CPRO-1 plants were significantly lower than on the Pikante Reuzen plants ($P<0.05$).

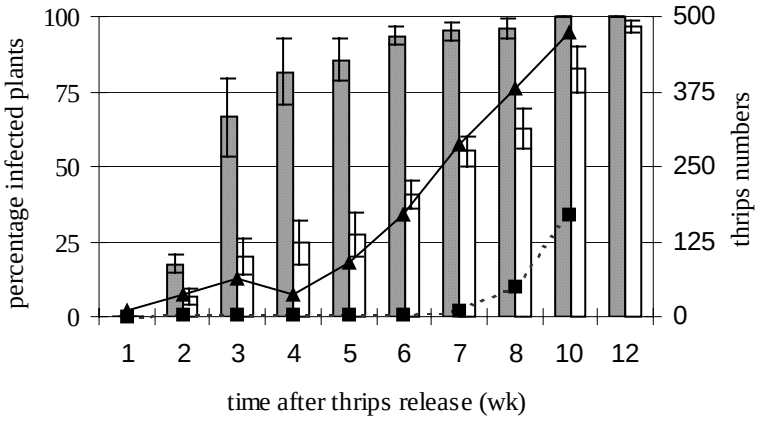

Figure 1. The development of a TSWV infection in virus susceptible plants of a thrips-resistant (white bars) and susceptible (grey bars) accession after the release of viruliferous *F. occidentalis* thrips in plots with plants of only one accession (non-choice plots). Lines depict the thrips population development on the thrips susceptible (solid line with triangle) or thrips-resistant (dashed line with squares) plants. Error-bars indicate the standard errors of the mean.

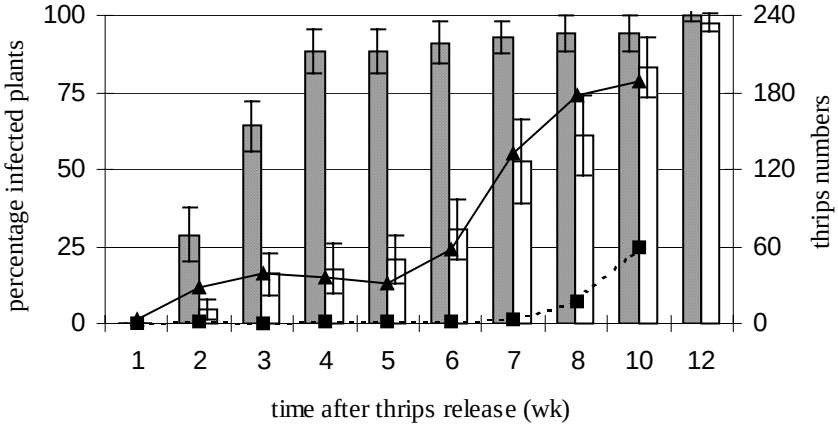

Figure 2. The development of a TSWV infection in virus susceptible plants of a thrips-resistant (white bars) and susceptible (grey bars) accession after the release of viruliferous *F. occidentalis* thrips in plots with plants of both accessions (choice-plots). Lines depict the thrips population development on thrips susceptible (solid line with triangle) or thrips-resistant (dashed line with squares) plants. Error-bars indicate the standard errors of the mean.

Reduced local lesion and thrips development in virus-resistant pepper

In the previous paragraph the effect of vector resistance on the spread of TSWV in virus susceptible pepper accessions was studied. The use of virus-resistant plants will be the favoured way to control virus infections in crops. This resistance trait is present in the thrips-resistant accession PI 152225 and the thrips susceptible accession PI 159236. After inoculation local lesions are produced on leaves and fruits (resulting in cosmetic damage) in consequence of a hypersensitive response. The effect of thrips resistance on the production of local lesions and the development of the thrips was assessed on these virus-resistant accessions.

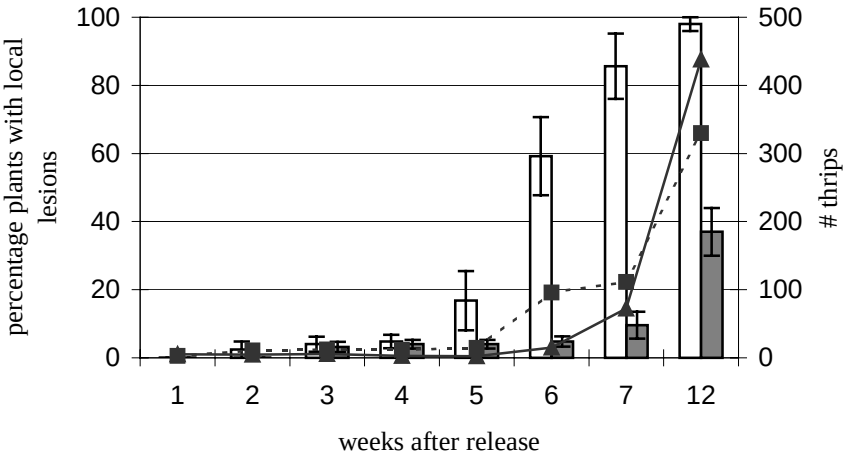

Figure 3. Development of local lesions on virus-resistant plants of a thrips-resistant (PI 152225; dark bars) and susceptible (PI 159236; white bars) accession after the release of viruliferous *F. occidentalis* thrips in plots with plants of only one accession (non-choice plots). Lines depict the thrips population development on the PI 152225 (solid line with triangles) or PI 159236 (dashed line with squares) plants. Error-bars indicate the standard errors of the mean.

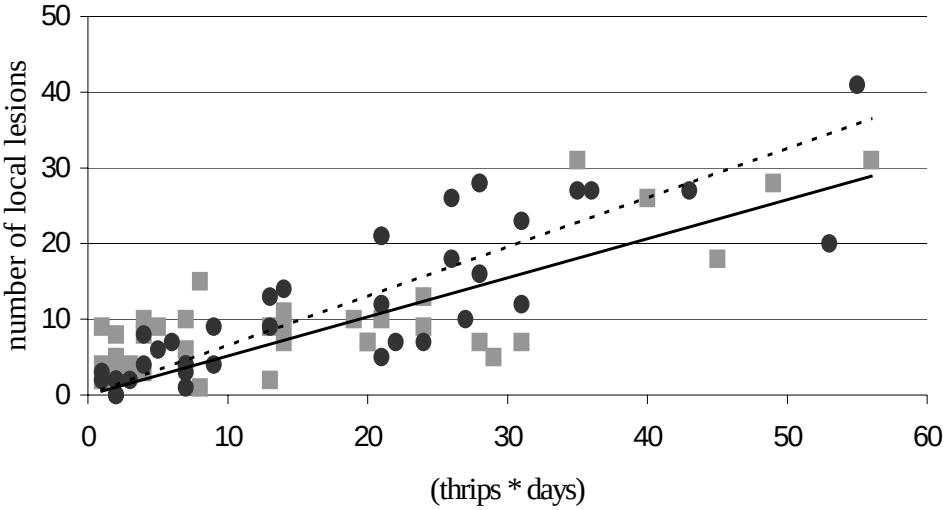

Figure 4. Infection of PI 152225 and PI 159236 plants by thrips confined on individual plants. The thrips population is expressed as the product of thrips numbers released on the plants and the number of days they could visually be recovered (thrips * days). Number of local lesions on PI 152225 plants: black dots, on PI 159236 plants: grey squares. Lines depict regression curves of the infection for the PI 152225 (dashed line) and the PI 159236 (solid line) accession.

The percentage of plants with local lesions developed at a significantly higher rate on the PI 159236 than on the PI 152225 plants (Fig. 3). The average of 136 lesions per PI 159236 plot was significantly higher than the 4 lesions per PI 152225 plot after 7 weeks. Only few thrips could be observed on both accessions during the first 7 weeks after their release but their number increased rapidly after the appearance of the first flowers (Fig. 3). The number of thrips observed on the PI 152225 plants was only significantly lower in week 6 after the onset of this experiment ($P<0.05$).

Comparing the data of Fig. 3 with those of Figs. 1 and 2 indicates that the rate at which the virus-resistant accessions became infected was considerably lower than for both virus susceptible accessions.

Effect of thrips resistance in virus-resistant accessions.

Plants of both virus-resistant accessions PI 152225 and PI 159236 were exposed to increasing numbers of viruliferous thrips to analyse the relation between the number of thrips and the number of local lesions produced. The number of local lesions in both accessions appeared to be almost similar when the plants were individually infested with the same number of thrips (Fig. 4). Possible differences in thrips mortality on both accessions were corrected by relating the number of local lesions produced to the product of thrips numbers released on the plants and the number of days they could visually be recovered (thrips*days; Fig. 4).

DISCUSSION

The influence of thrips resistance on both the spread of TSWV and the population development of *F. occidentalis* was analysed in a set of pepper accessions differing in their resistance to either the vector or the virus. This study was made on a semi-large scale in a greenhouse compartment containing the four pepper accessions, and in which thrips could freely move from plots with one accession to the other. A primary and secondary infection phase can be distinguished after the introduction of cohorts of viruliferous thrips. The primary infections are the result of the inoculations made by the released thrips at the start of the experiment and the secondary infections by the offspring of the released thrips. At the onset of the primary phase, a uniform infection pressure may have existed following the release of equal numbers of thrips in each plot. On application of such an infection pressure it could be expected that the same number of vector-resistant and susceptible plants would become primary infected, as these plants are equally susceptible to TSWV (Maris *et al*., 2003). However, the incidence of primary infected plants was significantly lower in the vector-resistant CPRO-1 plots than in the vector-susceptible Pikante Reuzen plots. This lower initial infection might indicate that either the thrips rapidly left the thrips-resistant plants before making a successful inoculation, or that when they do not move to the Pikante Reuzen plants, other factors negatively affect the successful inoculation of the CPRO-1 plants. In a previous study using leaf disks it has been demonstrated that thrips resistance hardly affects the inoculation efficiency (Maris *et al*., 2003). However, in that study the thrips were more or less forced to feed, and thus to inoculate the disks. In the present experiment the thrips could freely move and were not forced to feed on the resistant plants, which might explain the difference between the results from these experiments.

It remains unknown from the present results whether thrips differ in feeding behaviour on thrips susceptible and resistant plants resulting in less feeding scars and less virus inoculations on the latter. Evidence for an altered feeding behaviour on resistant plants was shown in other studies, *i.e.* for plants resistant to leafhoppers (Khan & Saxena, 1985), aphids (Haniotakis & Lange, 1974) or thrips (Harrewijn *et al.*, 1996). Leafhoppers made more probes on resistant cultivars (Khan and Saxena, 1985), while a greater restlessness of thrips was observed on resistant than on susceptible cucumber plants (Harrewijn *et al.*, 1996). This altered feeding behaviour may also result in more virus spread as shown in studies on chrysanthemum resistant to thrips (van de Wetering, 1999) and with aphids in resistant potato (Baerecke, 1958).

Since the first larvae can acquire virus from the systemically infected CPRO-1 and Pikante Reuzen plants approximately 8 days after the release of thrips, symptoms of secondary spread of TSWV can be expected 3 weeks later. The incidence progressed in the secondary phase at a significant lower rate on the thrips-resistant CPRO-1 than on the susceptible Pikante Reuzen

plants, although almost all plants became finally infected (Figs. 1 and 2). This difference in virus spread has to be explained by the poor reproduction or absence of any thrips reproduction on the CPRO-1 plants (Maris *et al.*, 2003). The increase, although slow, of the number of infected CPRO-1 plants will be the result of a dispersal of thrips from the Pikante Reuzen to the CPRO-1 plots. This dispersal does not result in similar numbers of thrips in both plots (Figs. 1 and 2) by which the incidence of infected plants in the CPRO-1 plots remains lower. The absence of an equilibrium in thrips numbers on the CPRO-1 and Pikante Reuzen plants also demonstrates that the dispersal between the plots was rather restricted or that the thrips moved more frequently from the CPRO-1 to the Pikante Reuzen plants than vice versa.

To assess whether thrips resistance would result in a decreased local lesion induction and therefore less cosmetic damage on fruits in TSWV-resistant pepper, spread in plots with two TSWV-resistant accessions (PI 152225 and PI 159236) was also studied. Besides the considerable lower number of thrips-resistant PI 152225 plants showing local lesions (Fig. 3), the number of local lesions produced per plot on the leaves of the resistant plants was also significantly lower than on PI 159236 plants. The difference in the number of infected PI plants and local lesions can not be explained by the slightly higher thrips numbers on the PI 159236 plants (Fig. 3). Moreover, a difference in susceptibility for TSWV can be excluded, as mechanical inoculation of both PI accessions produced similar numbers of local lesions when the same inocula were used (data not shown). Both accessions appeared also to be equally susceptible in tests in which thrips were confined to single plants of either accession (Fig. 4). The similar susceptibility of both accessions after thrips mediated virus inoculation and the large difference between the number of infected plants in the greenhouse indicate that other factors than population density play a large role in the spread of TSWV when the thrips have free access to plants. Whether a different feeding behaviour of thrips on plants of the different PI accessions and/or movement of thrips between these plants are involved, as discussed for the TSWV-susceptible accessions, remains unknown.

In this experiment, thrips resistance was found to have a significant positive effect on reducing virus spread in both virus susceptible accessions, resulting in low numbers of infected plants, and in virus-resistant accessions, resulting in less cosmetic damage. One of the main factors responsible for the reduced virus spread in the thrips-resistant accessions was the low thrips reproduction on and low preference for plants of these accessions, but these factors can not completely explain the low primary infections and the delay of the infection of the thrips-resistant plants. Nevertheless, the results show that thrips resistance will be a useful tool in IPM programs to control TSWV spread, in combination with cultural measurements, *e.g.* using thrips and virus-free stock material, biological control of thrips and removing virus-infected plants.

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Using mature breeding trees to induce infestations by the pine shoot beetle, *Tomicus piniperda* (Coleoptera, Scolytidae) in young pine trees

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Many decades ago the Netherlands installed a regulation by law, for obliged forest sanitation, to prevent outbreaks by bark beetles such as *Tomicus piniperda*. Felled timber should be removed from the forest before a certain date, in order to prevent the built-up of *Tomicus* populations. However, these regulations have negative side effects: they must be respected and supervised which is very costly. Another negative aspect is that felling and transports of trees is limited to a certain period. We have designed an experiment to estimate the likeliness of *Tomicus*-damage to young trees when populations of beetles are high. During a 5-year experiment we have tried to induce artificially infestations by laying felled mature breeding trees close to young pine stands, in order to monitor and to quantify the damage to the young trees. The insect infestations were counted on the top-lateral shoots and the leader top shoots. We did not succeed in raising the populations of *T. piniperda*, and by that, inducing infestations of this beetle in the young stands. However, the young trees suffered considerable attacks by *Rhyacionia buoliana*, and *Blastesthia turrionella*. These infestations, not caused by the breeding trees, could not be regarded as a real economical threat, because the main stems of most trees recovered. Considering the results of the experiments and the current forest management practices, such as natural forest regeneration, the constraining regulations for the Dutch forest management, are not continued anymore. Only if calamities should occur, the possibility exists to reactivate the regulations.

Keywords: bark beetle, forest sanitation, pine trees, *Tomicus piniperda*, *Rhyacionia buoliana*, *Blastesthia turrionella*

The Pine shoot beetle, *Tomicus piniperda* is regarded as a noxious insect for pine trees in Europe, northern Africa and Asia. This beetle breeds in the thick bark of trunks that have been felled, blown down or have died during the previous six months. The female beetle excavates the mother gallery and lays her eggs along both sides. The larvae excavate their tunnels and pupate at the end of the gallery. After emerging through the bark, the beetles fly up to the pine crowns in the surrounding area, where they bore into and tunnel up the centre of the current year's shoots. If there are strong winds, the shoots drop to the ground while still green. After this maturation feeding, the beetles of the new generation will overwinter in the hollow shoots or in short tunnels at the base of pine trees until the following spring. The beetles may be already active in February or March (Amezaga, 1997; Bakke, 1968).

The breeding can only succeed in recently dead or almost deceased trees. In general, vital full-grown trees cannot be killed. However, high populations of this beetle and the subsequent maturation feeding can reduce tree growth when the trees loose large numbers of shoots (Borkowski, 2001). In contrast with some decades ago, this kind of damage is regarded as no longer important and as an integral part of the natural processes. However, from literature it is known that also young trees can be attacked. In a 2 m high pine plantation, 61% of the trees were attacked in the upper parts (Turcek, 1964). If the leader shoots are attacked and break off, this could lead to malformations. When the young trees should form dichotomous trunks, the marketable length of the future timber wood is decreased. This can be regarded as an economical risk, because of the loss of wood quality and timber prices.

Many decades ago the Netherlands installed regulations by law, such as obliged forest sanitation, to prevent outbreaks by bark beetles such as *T. piniperda* and *Ips typographus*. These regulations include traditional silvicultural practices, such as forest sanitation by removing felled trees from the forest before mid-May, etc. However, these regulations have negative side effects: they must be respected and supervised which is very costly, both for the forest manager and for law enforcement. Another negative aspect is that the felling of trees and the transport to factories

etc. is limited to a certain period. This reduces modern flexible business procedures. Therefore it was suggested to freeze these regulations. The Dutch governmental forest organisation Bosschap asked us to conduct an experiment to estimate the likeliness of *Tomicus*-damage at young trees when populations of beetles are high.

In the following experiment we have tried to artificially induce infestations by laying felled mature breeding trees close to young pine stands, in the right condition to attract breeding insects, in order to measure and monitor the damage to the young trees caused by the next generations of beetles.

MATERIAL AND METHODS

Location and plots

In the pine forest area 'Boksenberg' near Someren, in each of 13 plots, a mature pine stand was situated adjacent to a young pine stand (Fig. 1). So each plot was divided in a mature and a young stand. The age of the mature stands varied from 48-68 years, that of the young stands from 9-10 years. Nine of these plots were treated with 5 mature felled trees. These trees were laid along the forest roads in-between the young and the mature stands to serve as breeding trees. This was done each year from 1995-2000. During the years 1997-2000, besides the 5 logs, another extra 1.5 m^3 of logs were deposited. These logs were felled each December to get suitable breeding trees. The purpose of these breeding trees was to raise the beetle population and by this to induce a local outbreak, in order to study infestations in the young trees. The remaining 4 plots served as untreated control plots. In each of the 13 young pine plots, experimental fields of 50 x 50 m were measured out for the quantification of infestations.

Estimating beetle populations

A fair method to estimate population densities, is to count the number of the beetles' exit holes in the breeding trees. However, this method is very labour intensive and too expensive. Counting the number of fallen shoots, due to the pruning by the beetles, also gives a fair estimation of the relative population densities (Doom & Luitjes, 1971).

Figure 1. Example of an experimental plot, with five mature felled trees along a forest road in between a mature pine stand and an adjacent young pine stand.

Table 1. The mean number of fallen shoots in the mature pine stands per m^2 from 1996-2000.

Treatment	Dec. 96	Apr. 97	Dec. 97	Apr. 98	Dec. 98	Apr. 99	Dec. 99	Apr. 00	Mean
Treated plots (n=9)	0.6	1.4	0.3	0.3	1.0	0.5	0.6	0.6	0.7
Untreated plots (n=4)	0.6	1.3	0.2	0.2	0.7	0.5	0.5	0.5	0.5

Table 2. Infestations of top-lateral shoots in young pine stands.

year	treatment	total number top-lateral shoots	*T. piniperda* (%)	*R. buoliana* (%)	*B. turrionella* (%)	infestation unknown (%)
1996	treated	7.403	0	1.9	1.0	3.1
	untreated	2.603	0	6.0	4.5	1.8
1997	treated	8.931	0	1.5	7.9	1.7
	untreated	3.369	0	2.5	7.0	0.3
1998	treated	11.817	0	0	0.6	0.4
	untreated	4.691	0	0	2.0	2.0
1999	treated	10.168	0	0.1	0.2	0.3
	untreated	4.436	0	0.3	1.3	0.5

Table 3. Total infestations of top shoots, in the young pine stands, by both *R. buoliana* and *B. turrionella*.

year	treatment	total number leader top shoots	% infested leader top shoots	s.d.
1997	treated	1399	6.9	2.7
	untreated	543	7.1	1.8
1998	treated	1418	2.8	1.7
	untreated	578	2.1	2.2
1999	treated	1359	3.4	0.8
	untreated	588	3.7	1.3
2000	treated	1379	3.4	1.5
	untreated	560	3.4	2.2

The fallen shoots were counted each year, both in April and in December, in 15 random permanent square meters, in each of the 13 corresponding plots in the mature stands. The first counting was carried out at the start of the experiment in December 1996, the last in April 2000.

Quantification of damage at young trees

In each of the 13 young pine plots, every year in July-August, a total of about 175 trees in 5 random rows was observed for infestations. Besides infestations by the Pine shoot beetle, *T. piniperda*, the trees may also suffer attacks by Pine shoot moth, *Rhyacionia buoliana*, and the Pine bud moth, *Blastesthia turrionella*. The infestations of these insects were determined by the external symptoms of their specific damage. These infestations were counted, as a total for both insects, for the top-lateral shoots and the leader top shoots. At the end of the 5-year experiment the global quality of the young stands were briefly evaluated by the forest manager.

RESULTS

Estimating beetle populations

The relative beetle populations were estimated by counting the fallen shoots in April and December. The results in Table 1 show that in April 1997, an increase in the amount of fallen shoots is observed for the treated as well the untreated plots. This increase is most likely due to high wind speeds and not to the beetles' activity. In 1999 and 2000, the mean numbers of shoots were of the same level as at the start of the experiment in 1996. It is concluded that the use of breeding stems did not result in inducing higher beetle populations in the treated plots.

Quantification of damage at young trees

The insect infestations were counted for the top-lateral shoots (Table 2) and the leader top shoots (Table 3). From Table 2 it can be concluded that infestations by *T. piniperda* were not observed at all, while *R. buoliana* and *B. turrionella* caused a considerable number of infestations in 1996 and 1997, often more in the untreated stands. Table 3 shows only low variations in the percentages of infested shoots in both the treated and untreated trees. These variations are not significant. The infestations of the leader top shoots were not caused by *T. piniperda* but by *R. buoliana*, and *B. turrionella* (see Table 2). At the end of the 5-year experiment, the quality of the young stands was estimated globally. The mean height varied from about 6-7 m and the trees scored from average to good form quality. Despite the heavy infestations by *R. buoliana* in 1997, the trees have recovered with a good tree shape.

DISCUSSION

In the Netherlands, high beetle populations have been reported after storms when breeding material such as pine logging slash has been abundant. On some locations averages of 15 - 56 fallen shoots per m^2 were counted during high infestations (Doom & Luitjes, 1971). However, in normal situations in sanitary managed forests, the average of fallen shoots is less then 1 shoot per m^2 (Nilsson, 1974; Michalski & Witkowski, 1962; Långström & Hellqvist, 1990). During our 5-year experiment, we did not succeed in raising the beetle populations. It was therefore not possible to induce any infestations in the young trees. From literature it is known that the beetles are capable to infest young trees. However, this happens only in the cases that large amounts of felled mature trees are stored in the vicinity of young pine stands for a long time (Hanson, 1937; Turcek, 1964).

Apparently really large quantities of stored trunks or storm-felled wood are necessary to increase the beetle populations. Because blue staining by fungi of the wood can occur rapidly, the wood is sold and removed quickly to avoid a low quality, which results in lower prices of the wood. This is a commercial stimulus that will result in low population densities of the beetle.

We did not succeed in raising the populations of *T. piniperda*, and by that, inducing infestations of this beetle in the young stands. However, the young trees suffered attacks by *R. buoliana*, and *B. turrionella*. These infestations could not be regarded as a real economical threat, because the main stems of most trees recovered.

Considering the results of the experiments, the today's diversified forest composition, the small-sized forests, and the current forest management practices (natural forest regeneration), it cannot be justified to keep imposing the constraining regulations for the Dutch forest management. It is recommended to avoid large wood yards during a long period in the vicinity of pine stands. However, this situation is unlikely to happen because of the economic necessity to remove the felled stems as quickly as possible to avoid an unwanted blue staining. Only if calamities should occur, the possibility should still exist to reactivate these regulations again.

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Manipulating biodiversity in arable farming for better pest suppression: which species and what scale?

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Field margins can attract and conserve predators and parasitoids, and may thus contribute to pest suppression. On 20 hectares of organic farming, a network of field margins has been laid down to investigate three main questions: 1. Does an increased biodiversity in a farming system contribute to the suppression of pest populations? 2. How far can field margins be apart? 3. What vegetation diversity is required in order to achieve pest populations suppression? Pitfall traps and yellow water pans in field margins and crops are used to assess antagonists' densities. Samples of key pests and damage assessments in different crops are related to the distance from the nearest margins. The first results out of these massive data sets are presented. In both wheat and potato, aphid incidence appeared unrelated to the distance from the nearest crop edge. However, aphid densities were much lower in the BIOdivers subsystem with field margins compared to the control system. A similar pattern was observed for carrot fly (*Psila rosae*; Diptera, Psilidae) damage in carrots. This reoccurring pattern suggests that in the field margin network of the BIOdivers system, the total assemblage of (generalists and specialists) predators and parasitoids exert a degree of pest suppression in several crops, over distances of at least 50 m.

Keywords: field margins, functional biodiversity, biological control, farming systems, Carabidae, spatial variation

During the last decades, biodiversity in agricultural landscapes in Western Europe has declined considerably. Road verges, watercourses and field margins have become the dominant refugia for biodiversity in agricultural landscapes. Consequently, the role of field margins in the conservation of plants, birds, mammals, butterflies and other groups has received a lot of attention (*e.g.* Boatman, 1994; Boatman *et al.*, 1999; Tamis *et al.*, 2001). Among many functions (Marshall & Moonen, 2002), field margins may play an important role in conserving pollinators, generalist predators and parasitoids, and may contribute to substantial degrees of natural control of agricultural pests in adjacent field crops (*e.g.* Thomas *et al.*, 1992; Meek *et al.*, 2002; Collins *et al.*, 2002).

In the open landscape of the Dutch Noordoostpolder, with very few natural landscape elements, we started a large-scale field experiment to investigate whether field margins can attract and conserve predators and parasitoids, and thus contribute to pest suppression. A network of permanent field margins sown with grass and perennials has been laid down on an organic farm to investigate three main questions: 1. Does an increased biodiversity in a farming system contribute to the suppression of pest populations? 2. What is the optimal or maximal distance between field margins? 3. What vegetation diversity is required in order to achieve pest populations suppression?

MATERIAL AND METHODS

At an experimental farm in Nagele, an organic farming system with a 6-year crop rotation of potato, summer wheat, iceberg lettuce, carrots, white cabbage and grass-clover is being studied. On a 10-hectare subsystem, hence called 'BIOdivers', a network of permanent field margins sown with different grass and wild flower mixtures has been laid down in spring 2001. Field margins were laid down in such a way that six fields were created (one for each crop) as follows (also see Fig. 1A): two large fields of 110 x 130 m, another two large fields which are subdivided each by field margins into four smaller plots of 50 x 60m, and the last two fields that are divided each by field margins into eight smaller plots of 36 x 50 m. Field margin width was varied in such a way that the surface ratio of plot versus surrounding margin remains constant. The East-West axis represents the network intensity gradient, and should clarify the maximum distance over which arthropod predators and parasitoids significantly reduce pest population densities. Field margins were sown and managed in such a way that a vegetation diversity gradient was created along the

North-South axis (Fig. 1A). This gradient is used to clarify how diverse field margins should be in order to host sufficient antagonists to reduce pest population densities. Theoretically, a biodiversity gradient towards the Northeast corner of the system will develop over the years, and insight will be gained in the optimal combination of margin heterogeneity and network intensity for pest suppression.

A second subsystem of 10 hectares (called 'BIOintensief') (Fig. 1B) has six large parcels (one for each crop) and has three narrow field margins along the top, middle and bottom of the subsystem, in East-West direction. This system serves as a reference ('control') system, in order to correct for background infestation levels of mobile (airborne) pest densities. Since both systems are not replicated, effects will be analysed with crops and years as variables.

Pitfall traps and yellow water pans, in use from mid-May until mid-October, were used to collect samples of the natural enemies in the systems. Pitfall sampling took place by sampling 80 locations distributed over both systems (see Fig. 3 for indication of locations) (three pitfalls per location, 5 m apart, combined into one sample). A total of 30 yellow water pans were distributed over both systems, but trapping results were very poor and will not be discussed in this paper. Pitfalls were filled with a 5% formaldehyde solution and emptied every 14 days. Catches were stored in 70% ethyl-alcohol at 5°C and were sorted and counted in the laboratory into functional groups (mostly at the order or family level).

Key pests, such as aphids (Homoptera, Aphididae), caterpillars (Lepidoptera, several families), root flies (Diptera, Psilidae), leaf beetles (Coleoptera, Chrysomelidae) and slugs (Mollusca, several families) were selected for each crop, and optimal sampling periods and methods were chosen for each pest. In 2002, pest densities (numbers), incidence (presence/absence per plant or shoot) or damage levels were assessed at 2, 15 and 50 m into the crop from the nearest field margin or crop edge.

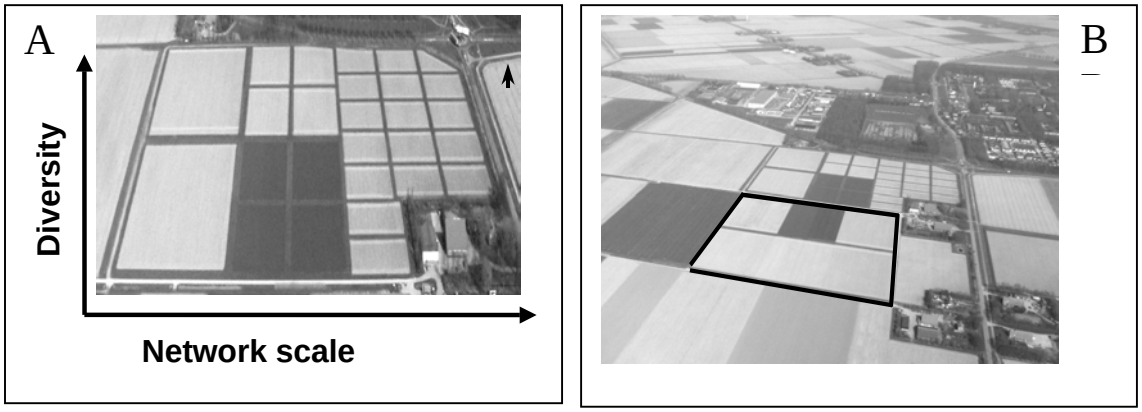

Figure 1. Aerial view of the experimental systems at Nagele. A. The BIOdivers subsystem with the network of field margins. B. The control system without a margins network (the black rectangle). Each subsystem is approx. 10 ha in surface; the top of the photographs is facing North.

RESULTS

To this date (December 2002), about 40% of the pitfall samples of the 2001 season have been sorted and counted (62800 arthropods sorted). Spiders (Araneae; predominantly dwarf spiders, Erigonidae) were the dominant group with 30400 specimens (48% of total catch). Carabid beetles (Coleoptera, Carabidae) were presented by 15900 individuals (25%) and rove beetles (Coleoptera, Staphylinidae) constituted 7900 (13%) of the catches. The pitfall samples of the 2002 season will be sorted and counted in January 2003, and could therefore not be included in this paper. Data of the pitfall traps presented here fall into two groups. The first group presents results from three sampling locations (in a white cabbage plot, in a field margin between the white cabbage field and

the carrot field, and in a grass-clover field) in the BIOdivers subsystem, that have been sorted for the whole 2001 sampling season (time series from mid-May until mid-October 2001). The second group represents two sampling periods (week 20-22, *i.e.* second half of May, and week 30-32, *i.e.* late July and early August 2001) in which all traps in the system were sorted (spatial overviews).

To illustrate the first findings in this experiment, we present the trapping results for carabid beetles (Fig. 2A) and spiders (Fig. 2B) as a time series throughout the 2001 sampling season (May-October) from three locations in the BIOdivers subsystem. Carabid beetles (Fig. 2A) seemed to prefer the open plots of white cabbage above the densely grown grass-clover plots and grassy field margins. In contrast, spiders (Fig. 2B) were more abundant in the field margins and grass-clover plot than in white cabbage field.

An example of the spatial variation in the systems is given in Fig. 3, for carabid beetles caught in August 2001. Highest carabid densities were found in the plot of white cabbage of the BIOdivers system (Fig. 3A, lower left corner).

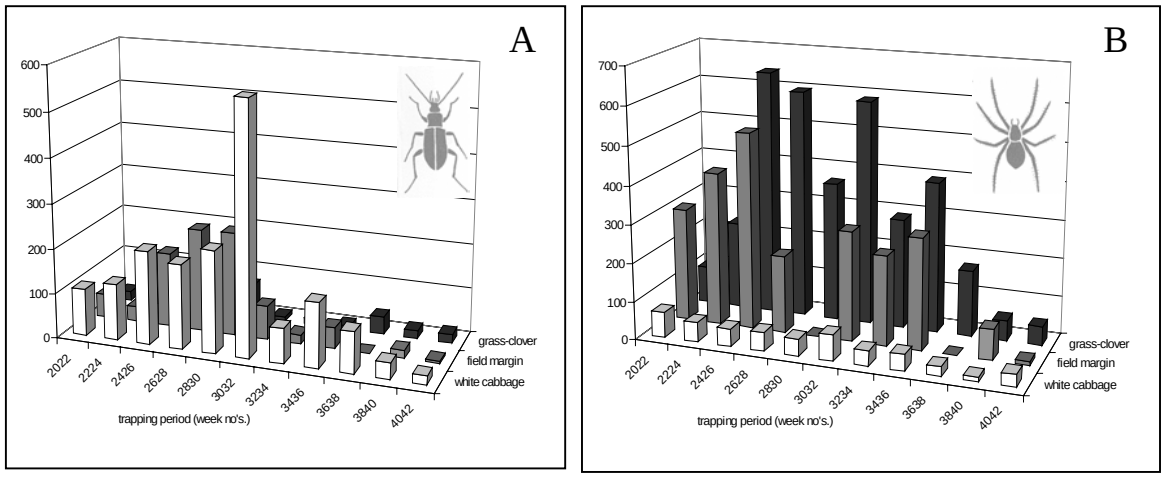

Figure 2. Results from 3 pitfall traps in the BIOdivers subsystem (+ margins) during the 2001 trapping season (11 biweekly samples from week 20, early May, until week 43, end October). A: Carabid beetles. B: Spiders.

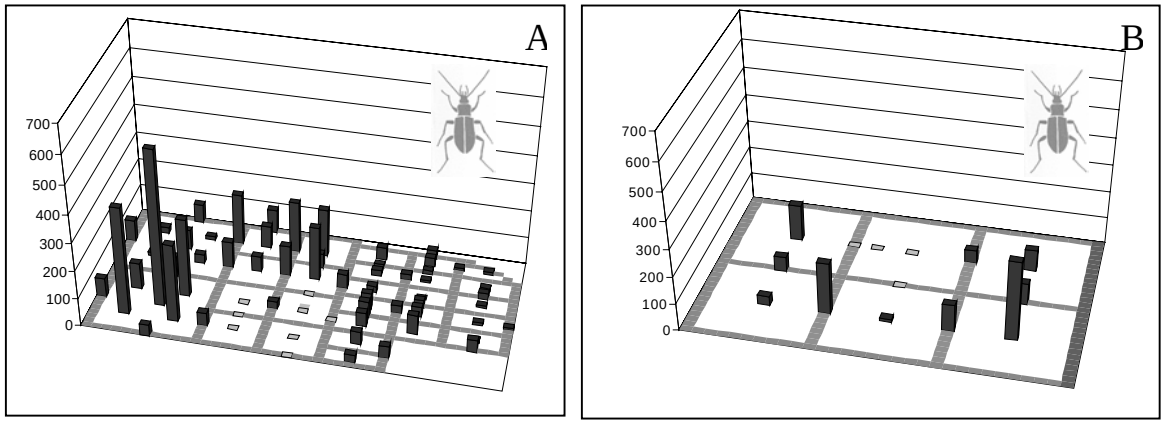

Figure 3. The numbers of carabid beetles caught in each pitfall trap in week no. 30-32, August 2001. A: in the BIOdivers subsystem (+ margins) and B: in the BIOintensief subsystem (no margins).

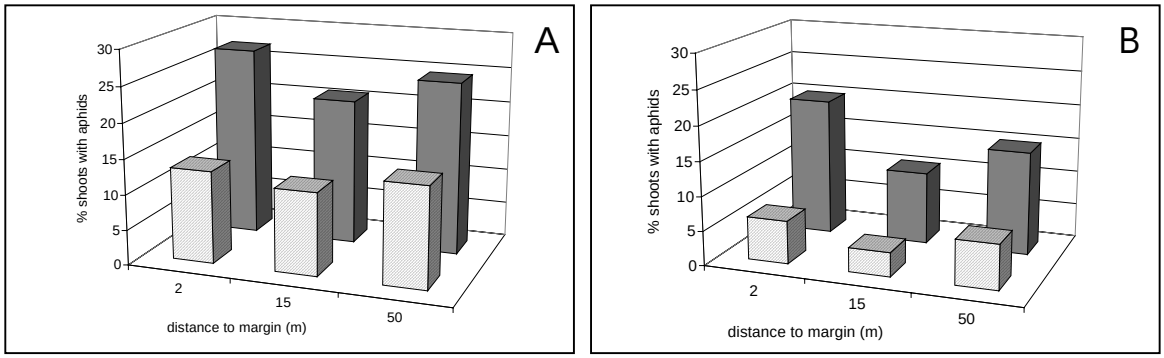

Figure 4. Percentages of shoots infested by aphids at different locations within plots in relation to the distance to the nearest crop edge. Hatched columns: in the BIOdivers subsystem (+ margins); filled grey columns: in the BIOintensief subsystem (no margins). A: in summer wheat, June 2002 and B: in potatoes, July 2002.

Sampling of key pests revealed lower levels of leaf-feeding beetle (*Lema* sp.; Coleoptera, Chrysomelidae) damage along the margins of summer wheat, compared to the centre of large plots (data not shown). In contrast, in white cabbage and Brussels sprouts, the density of Diamond-back moth (*Plutella xylostella*; Lepidoptera, Plutellidae) appeared highest along field edges (data not shown).

In summer wheat, aphid incidence in June 2002 appeared unrelated to the distance to the nearest crop edge. However, aphid densities were much lower in the BIOdivers subsystem than in the BIOintensief system (Fig. 4A). A similar pattern was also observed for (other) aphids in potato in July (Fig. 4B) and for carrot fly (*Psila rosae*; Diptera, Psilidae) damage in carrots in October 2002 (data not shown).

DISCUSSION

The data presented here provide only the first glimpses of what is going on in this complex system. More time and effort, more seasons and further processing and analyses of data are required to answer our research questions. The 2001 season yielded approx. 150,000 arthropods in the pitfall traps alone, about 50% of them belonging to the spiders, 25% to the carabid beetles and 13% to the rove beetles. So far, only 40% of the 2001 samples have been sorted. Numbers (as an indication of activity) of these generalist predators vary widely in time and space, representing seasonal phenology but also distinct habitat preferences (Figs. 2A, 2B and 3A). Habitat preference may be influenced by vegetation structure, microclimate, prey abundance, agricultural activities, and other factors. The pitfall samples of the 2002 season will be sorted and counted in January 2003 and will improve our insight in the spatial and temporal dynamics of the functional groups in our experimental system.

In 2002 key pest populations were sampled for the first time in every crop. For only a few pests, density gradients in relation to the field margins were observed. Damage of leaf-feeding beetles (*Lema* sp.) was lower along the margins of summer wheat, compared to the centre of large plots (data not shown). This suggests pest suppression by predators (with a limited mobility) from the field margins. In contrast, in white cabbage and Brussels sprouts, the density of Diamond back moth (*P. xylostella*) appeared highest along field edges (results not shown).

An interesting pattern of aphid densities (for the total species complex) was found in summer wheat and potato. In both crops, aphid incidence appeared unrelated to the distance from the nearest crop edge. However, aphid densities were much lower in the BIOdivers subsystem compared to the BIOintensief system (Fig. 4A and B). A similar pattern was observed for carrot fly (P. rosae) damage in carrots. This reoccurring pattern suggests that in the field margin network of the BIOdivers system, the total assemblage of (generalists and specialists) predators and parasitoids exert a degree of pest suppression in several crops, over distances of at least 50 m.

Since most key pests were sampled only once or twice in the 2002 season and data of the antagonists monitoring are not yet available, causal relationships and underlying mechanisms cannot be analysed at present. In the 2003 season, pest sampling will be focussed on two or three key pests and crops only, and carried out with a (bi)weekly frequency, in order to get a better insight in population dynamics and rates of predation and parasitism. Possibly, exclusion experiments with barriers and cages to exclude antagonists at different distances from field margins, can be carried out to analyse the effects of pest suppression and the contributions of antagonists from the field margins. Marking and release experiments could demonstrate the dispersal distances of predators, and whether field margins act as sources or sinks for antagonists in crops. The BIOdivers field margin system thus offers great opportunities to study different hypotheses with regard to functional biodiversity in agricultural systems. We hope to set up co-operation with other research groups in order to tackle several of these interesting questions.

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Insect diversity on yellow Asteraceae in road verges in the Netherlands

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Yellow Asteraceae, which are very common in Dutch road verges, are known to attract many insect species. Insect visitors on three most common yellow Asteraceae flowers (*Hieracium laevigatum, Hypochaeris radicata* and *Leontodon autumnalis*) were studied. Over 80% of the insect species (mostly syrphids, and also some oligolectic bee species) visited at least one of those three plant species. Insect diversity (Shannon index) appeared to be positively related to flower diversity. In a flower constancy experiment *Eristalis tenax* discriminated flowers of two of the three plant species, presumably because those flowers differ in diameter size or UV-reflection.

Keywords: insect diversity, plant diversity, yellow Asteraceae, flower constancy

In agricultural landscapes many flowering plants can only survive in linear (habitat) fragments, like road verges and ditch banks. Besides their function as a refuge for plant species, road verges may also play an important role as corridor between nature areas, for both plants and animals. The management of those sites, which is often done by local farmers, can affect insect diversity, both directly and indirectly via plant diversity. Plant species flowering at the same time and place may interact for pollinator visits in a variety of ways: plants can have a facilitative function by attracting more pollinators (Rathcke, 1983). On the other hand, competition among flowers for pollinators is possible (Thomson, 1978; Kwak *et al.* 1998; Chittka & Schürkens, 2001).

The family of the Asteraceae is one of the largest families of flowering plants, with 25,000 species over the world (Proctor *et al.*, 1996). Asteraceae grow mostly in sunny (micro-)habitats. A relatively large number of Asteraceae can be found in grasslands and road verges, especially on dry to moderately wet habitats (Weeda *et al.*, 1988). The attractiveness of flowers for insects depends on floral traits, such as volume and sugar composition of nectar, and corolla tube length of flowers (Torres & Galetto, 2002). Most entomophilous plants are visited by a large variety of insects (Proctor *et al.*, 1996). Yellow Asteraceae have flowers in which nectar and pollen are easily accessible, so many insect species can visit these plant species. Insect visitors can be divided into two groups: the generalists (polylectic species) and the specialists (mono- and oligolectic species). Specialist insects can be found among the bees: almost one-third (76 out of 224) of bee species in the Netherlands are oligolectic (Peeters *et al.* 1999). Thirteen of the oligolectic bee species are specialists on Asteraceae.

In this paper we present data on the insect diversity on three yellow Asteraceae common in Dutch road verges: *Hieracium laevigatum, Hypochaeris radicata* and *Leontodon autumnalis.* The relation between insect and flower diversity was investigated. In addition we examined if insects can discriminate between flowers of different yellow Asteraceae species.

MATERIALS AND METHODS

Study area

The study was performed in the North of The Netherlands, near Assen. In the Drentsche Aa area five sites (road verges and ditch banks), near Bovensmilde four sites and near Ekehaar one site were selected.

Flower and insect diversity: transect observations

Flowering plants and insects were counted in transects of 100 by 1-1.5 meters at each site. Flowers of yellow Asteraceae appeared to close during the day, depending on site, date and species between 13.00 and 15.00 h. Therefore transect observations were carried out between 9.00 to 13.00 h.

Numbers of flowers of all flowering plant species interesting for insects were recorded one to three times at all sites. Depending on the plant species, single flowers or flowering units were counted (*e.g.* in Asteraceae the number of heads). Flower units are units that a medium-sized bee has to fly, rather than walk, between (following Dicks *et al.*, 2002). All flower-visiting insects in combination with the plant species were recorded one to five times at all sites, walking at a slow speed.

Plant species were identified using Van der Meijden (1995), if there was uncertainty about a species. Insects were recorded at species level as far as possible. When uncertainty about a certain species existed, individual insects were caught. Hoverflies were identified in the field, using Reemer (2000); bees (Apidae) were labelled for later identification. All insects longer than 3 mm were included. Smaller insects are easy to miss, they can be extremely difficult to identify while walking a transect, but first of all they cannot be comprehensively collected without disturbing insect-flower interactions (Dicks *et al.*, 2002). Insects were grouped in orders: Syrphidae, Diptera rest, Apidae and Lepidoptera.

Transect observation data of flowers and insects were used to calculate diversity, with the Shannon index of diversity (Magurran, 1991). This formula incorporates number of species as well as a measure of abundance. The relation between the diversity of insects and plants was investigated.

Flower constancy experiment

Heads of two of the three target species were experimentally arranged in a patch. All (three) combinations of two target species were examined. Test-tubes filled with water attached to wooden sticks were placed in the ground, in a way that the distances between each flower is equal, *i.e.* 10 cm. Therefore the plot had a total dimension of 40 by 35 cm. Visitation of individual insects was recorded and note was taken on changes between flowers. Observations of individual insects were summed for analysis, with a minimum of 15 transitions per insect species.

We used the following measure for flower constancy: the number of intraspecific transitions per plant species divided by the total number of transitions. This constancy has a range from 0 to 1. Insects can only show flower constancy if they can distinguish flowers of different species in a certain way. Therefore flower diameter size was measured of thirty flowering heads of each target species.

Statistics

The relation between insect diversity and flower diversity was analysed using linear regression. Possible differences between numbers of transitions in the flower constancy experiment were analysed with a χ^2 goodness of fit test.

RESULTS

Flower and insect diversity: transect observations

In total, 55 flowering species were recorded, of which 6 species of yellow Asteraceae. The number of flowering plant species varied from 4 to 20 species per transect, depending on site and date. The number of flowering units varied from 144 to 3187 per site. Depending on month and site, yellow Asteraceae flowers can make up to even 96% of all the flowers. The plant diversity, calculated with the Shannon index (H), varied from 0.48 to 1.96.

In total, 48 insect species were recorded. The number of insects species varied from 2 to 22 species per transect; the total number of insects varied from 3 to 79 individuals per transect. The insect diversity varied from 0.64 to 2.54. The number of insects, as well as the diversity declined in the season (R^2=0.16, p=0.01 and R^2=0.39, p<0.001, respectively). The insect diversity was positively correlated with the flower diversity (R^2=0.24; p=0.02) (Fig. 1).

The three target plant species were visited by many different insect species (Fig. 2), but mostly by Syrphidae species. We observed at least three oligolectic bee species specialised on yellow Asteraceae. Over 80% of all insects were observed to visit at least one of the three target plant species.

Flower constancy of insects: an experiment

The flowering heads in the experimental arrangement were visited by a range of different insects. However, only 3 out of 19 visiting insect species made more than 15 transitions. *Eristalis tenax* showed a significant flower constancy for *H. laevigatum*, when it had a choice between *H. laevigatum* and *L. autumnalis* (χ^2=5.18; df=1; p<0.05). When *E. tenax* could choose between *L. autumnalis* and *H. radicata* it preferred the first one (χ^2=20.38; df=1; p<0.05) (Table 1). Other insect tested showed no significant discrimination behaviour.

Table 1. Number of visited flowers per offered combination and flower constancy for three insect species.

Insect species	flower combination	# visited flowers	sign.	flower constancy		sign.
Eristalis tenax	HL-HR	12-19	ns	0.04	0.23	ns
E. tenax	HR-LA	1-15	<0.05	0	0.85	<0.05
E. tenax	HL-LA	55-36	<0.05	0.33	0.14	<0.05
Sphaerophoria sp.	HL-HR	13-11	ns	0.25	0.13	ns
Panurgus banksianus	HR-LA	10-13	ns	0.05	0.14	ns

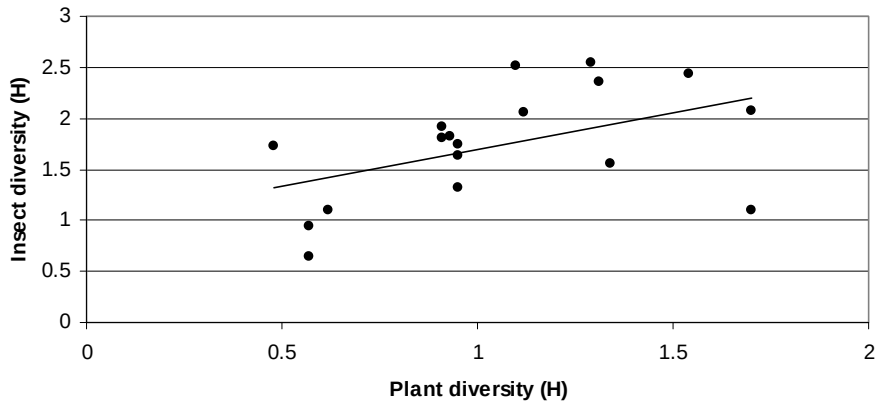

Figure 1. Insect diversity (Shannon index) as a function of flower diversity and regression line (y = 0.73x + 0.97; R^2 = 0.24; N = 18; p = 0.02)

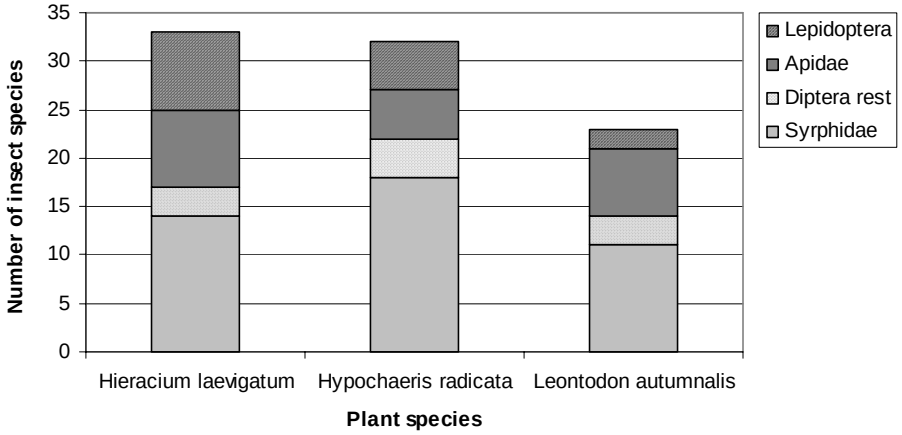

Figure 2. Number of insects species on three common yellow Asteraceae species.

DISCUSSION

Plant diversity differed between sites. Overall, insect and plant diversity were positively related. Also insect diversity differed largely between sites and decreased during the season. Part of the variation can be explained by the mowing regime. After mowing it took some time before plants flowered again.

Yellow Asteraceae are dominant flowering species in road verges. A high percentage (over 80%) of the insect species observed during transect observations were recorded at least on one of the three common yellow Asteraceae species in road verges. The three investigated plant species did not differ in insect species composition. Syrphidae were the largest visitor group, however, they were also frequently seen on other plant species like *Valeriana officinalis*. Specialist bees have no alternative but to forage on yellow Asteraceae.

Eristalis tenax appeared to be the only insect species to show a significant flower constancy. Flower constancy of syrphids has rarely been shown (Goulson & Wright, 1997). It can be said that *E. tenax* is able to discriminate between the three target plant species, although not when the combination *H. laevigatum-H. radicata* was offered. Except for the diameter of the flowers, which was smaller for *L. autumnalis* than for the other two plant species, insects can also discriminate between flowers due to their difference in UV-reflection. The yellow heads of *H. laevigatum* do reflect UV radiation, whereas the heads of *L. autumnalis* do not (L. Chittka, pers. comm.). No data on the UV-reflection of the third species, *H. laeviagtum* were found, but since *E. tenax* did not discriminate between flowers of this species and flowers of *H. radicata* we presume that *H. leavigatum* does also not reflect UV radiation.

Other insect species did not choose one of the two target plant species over the other. However, this can also mean that those plant species had the same reward at that moment, which means that there was no reason to discriminate between the flowers.

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Malathion Resistance in *Drosophila melanogaster* (Diptera: Drosophilidae)

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Malathion resistance developed in a *Drosophila melanogaster* population that has been exposed to malathion for almost 20 years. Genetic analyses showed that malathion resistance in this population is under control of several genes that are located on the first, second and third chromosome. The second chromosome contributes the most to resistance; the factor(s) involved may be (partly) dominant or additive and additional recessive genes may also be present on this chromosome. Although to a lesser extent, the third chromosome also contributes to resistance, the factors being mostly recessive or only expressed in interaction with the second chromosome. On the X-chromosome, a factor with a very small effect on resistance was observed.

Keywords: *Drosophila melanogaster*, insecticide resistance, malathion, localisation

During the second half of the last century, synthetic insecticides were intensively used to control pest insects (Casida & Quistad, 1998). However, in many cases the treated pest insects rapidly developed resistance to the applied insecticides. Consequently, development of resistance has become a significant problem for pest management in agriculture. Understanding the evolution and genetic basis of resistance, therefore, is of utmost importance in this applied field.

The general aim of our project is to determine the genes involved in insecticide resistance in *Drosophila melanogaster* populations that for a long period (nearly 20 years) have been treated with insecticides and particularly to study the possible evolution of modifier genes that decrease the cost of resistance. Before we can study the evolution of modifiers, we first have to locate the primary genes responsible for resistance. This is the aim of this present study, where we focus especially on malathion resistance. The organophosphate malathion was applied in 1952 for the first time (Casida & Quistad, 1998). With other organophosphates, such as trichlorfon and dimethoate, malathion belongs to the aliphatic derivatives, which are compounds with straight carbon chains (Pedigo, 1989). In *D. melanogaster*, previous studies have indicated that malathion resistance is under polygenic control (*e.g.* Halpern & Morton, 1987; Houpt *et al.*, 1988). In these studies, genetic factors affecting resistance were observed on both the second and third chromosome of *Drosophila*.

Here, we report the chromosomal localisation of genetic factors involved in malathion resistance by means of visible genetic markers and balancer chromosomes (King & Sømme, 1958; Hartl & Clark, 1997).

MATERIAL AND METHODS

The *Drosophila melanogaster* populations

From the base population '50 x 50' (see Bijlsma & van Delden, 1977, for description) two replicated cage populations were started in 1983. One of the offshoots (designated by **R** throughout the paper) was initially reared on food supplemented with 0.15 ppm malathion. This concentration was gradually increased until 1987, when a final concentration of 0.9 ppm was reached. From then on, the **R** population has been continuously reared on 0.9 ppm malathion. In 1987, the **R** population had already become highly resistant to malathion (R. Bijlsma & A.C. Boerema, unpublished). The other offshoot was maintained on normal medium and therefore likely to remain susceptible (designated by **S**) to malathion. As such, the latter population served as control.

The *Drosophila* populations (population size about 2000-5000 flies) have been reared in population cages (for description and construction of the cages see Beardmore *et al.* (1963)) at 25°C and 50-70% RH. In a rotating scheme, every week 5 of the 15 food cups in the cage were

replaced by fresh food cups containing 40 ml of standard medium consisting of 26 g dead yeast, 54 g sucrose, 17 g agar and 13 ml Nipagin solution (10 g Nipagin in 100 ml 96% ethanol) per 1000 ml water. For the population treated with malathion, malathion was added to the standard medium to a final concentration of 0.9 ppm. In all experiments, flies were cultured in an incubator room at 25°C and 50-70% RH.

Resistance measure

Malathion resistance was measured by determining egg-to-adult survival (viability) on normal (control) and malathion-supplemented medium. For each malathion concentration, 10 vials (10 cm high, Ø 1.5 cm, containing 8 ml medium) were established with 100 eggs each. All emerging flies from these vials were collected and the number of adults was determined.

RESULTS AND CONCLUSIONS

Sex-linkage and dominance of the factor(s)

Sex-linkage was investigated by assessing differences in viability between the sexes among the offspring from the crosses of **R** females with **S** males, cross **R** x **S**, and the reciprocal cross **S** females with **R** males, **S** x **R** (Fig. 1). The mean observed sex ratios were tested for deviation from the expected sex-ratio of 1 with a t-test. Table 1 shows no significant deviation from sex ratio = 1 for the **S** x **R** cross on all concentrations ($P>0.05$). However, for the **R** x **S** cross, on the two highest malathion concentrations tested, significantly more males survived (0.9 ppm: $P<0.02$; 1.5 ppm: $P<0.001$). The males from the **R** x **S** cross received their X-chromosome from their resistant mother, whereas the **S** x **R** males received their X-chromosome from their susceptible mother. No differences in chromosomal composition of the females were present. Since only the number of males in the F_1 of the **R** x **S** cross is different on higher malathion concentrations, a minor genetic factor affecting malathion resistance is located on the X-chromosome.

Comparing the viability of the above crosses to that of the parental susceptible (**S** x **S**) and resistant (**R** x **R**) stock, enables us to get some information of the degree of dominance of the resistance factors involved. Figure 2 shows that on 1.5 ppm malathion the resistant population still survives nearly as well as on control medium, whereas the susceptible population already suffers greatly from a low concentration of 0.5 ppm malathion. This clearly shows that the long-term exposure to malathion leads readily to resistance for this insecticide. Although on control medium the resistant population shows a significantly lower viability ($P<0.001$) than the susceptible population, it survives much better on increasing concentrations of malathion than the heterozygous offspring of the crosses **S** x **R** and **R** x **S**. On 0.9 and 1.5 ppm, the survival of the reciprocal crosses **S** x **R** and **R** x **S** was similar, but it was highly significantly lower than that of the resistant (**R** x **R**) parental stock. The fact that the resistance of reciprocal crosses is somewhere in between that of the parental stocks indicates that the factors involved in malathion resistance are not completely dominant nor completely recessive. This may mean that either most factors involved act in an additive fashion or that part of the genes involved are (partly) dominant while others are (partly) recessive.

Table 1. The mean (± s.e.) sex-ratios (♀♀ : ♂♂) of the crosses **S** x **R** and **R** x **S**. *=Significant deviation from sex-ratio = 1, tested with a t-test ($P<0.05$).

Malathion (ppm)	S x R	R x S
0	0.86 ± 0.063	1.02 ± 0.100
0.5	1.09 ± 0.053	1.13 ± 0.175
0.9	1.58 ± 0.454	0.63 ± 0.085*
1.5	0.68 ± 0.143	0.43 ± 0.095*

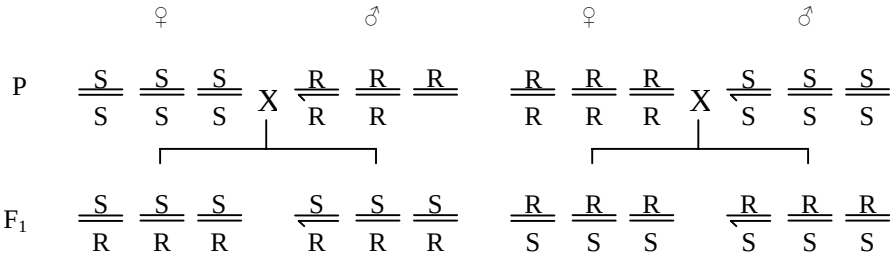

Figure 1. Crossing schemes to determine sex-linkage and dominance of the resistance factor(s). R designates chromosomes of the resistant strain, S those of the susceptible strain.

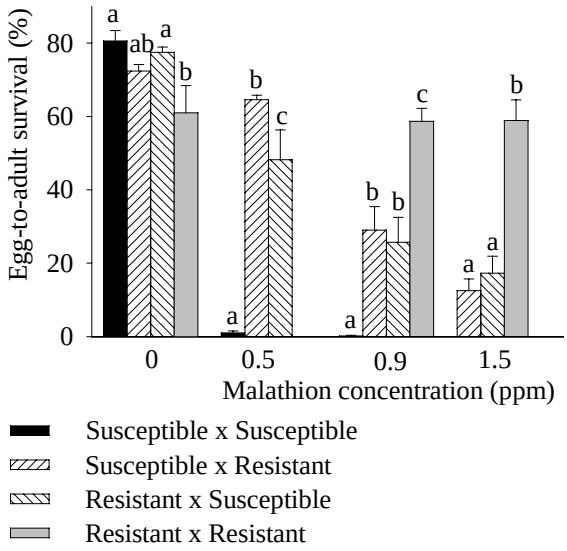

Figure 2. Mean egg-to-adult survival (+ s.e.) of the two reciprocal crosses and the two parental strains on different malathion concentrations to determine the degree of dominance of the resistance factor(s). The survival of Resistant strain (Resistant x Resistant) was not tested on 0.5 ppm. The survival of Susceptible strain (Susceptible x Susceptible) was not tested on 1.5 ppm. For each concentration different letters denote significant differences in mean survival between the different crosses. The same letter indicates no significant difference in survival on that concentration.

Dominant autosomal factor(s)

To determine the presence of dominant factors (collectively denoted by 'R' in Fig. 3) involved in malathion resistance on chromosome 2 and 3 (the small fourth chromosome of *D. melanogaster* was ignored), we crossed the **R** with the recessive *bw//bw;st//st* eye-colour marker strain (*bw*, brown eyes, and *st*, scarlet eyes, are located on chromosome 2 and 3, respectively). As depicted in Fig. 3, females of the (susceptible) marker strain were crossed with malathion resistant males. Next, F_1 males were backcrossed with *bw//bw;st//st* females. Egg-to-adult survival of the F_2 was determined for 3 malathion concentrations.

The survival of the four possible genotypes, that in the absence of malathion are expected in the ratio 1:1:1:1, is shown in Fig. 4. In our experimental design this means expected numbers of 25 for each genotype. Even though on control medium already some significant differences in survival are observed (probably due to the presence of the eye colour chromosomes), the most important finding is that the genotypes *bw//bw;st//st* and *bw//bw;st//*R do not survive on malathion

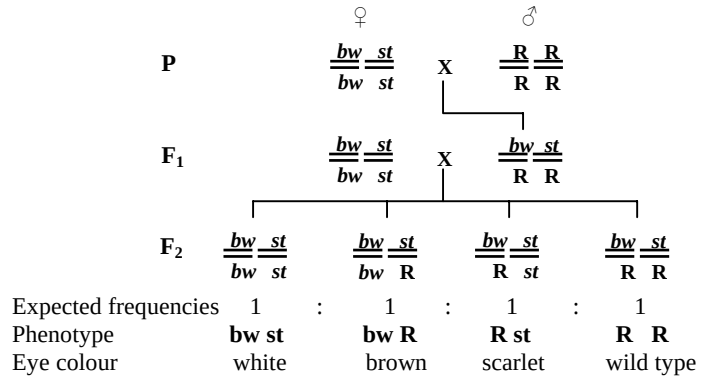

Figure 3. Crossing scheme to investigate the presence of dominant factor(s) on the autosomes by means of recessive markers. *bw*=brown eyes (chromosome 2); *st*=scarlet eyes (chromosome 3); the combination of both markers in homozygous condition produces white eyes.

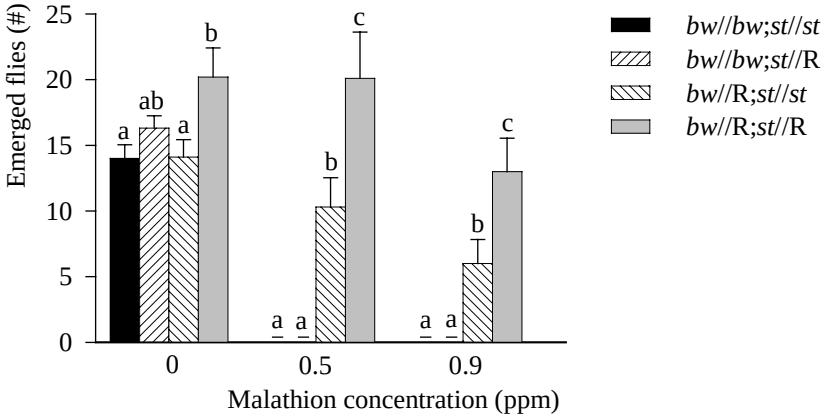

Figure 4. Mean egg-to-adult survival (+ s.e.) for different malathion concentrations. For each concentration, bars marked by the same letter indicate no significant difference in survival between genotypes for that concentration.

at all, whereas the genotypes *bw//R;st//*R and *bw//*R*;st//st* show high to moderate survival. This indicates that one or more (partly) dominant factors are located on the second chromosome. Moreover, survival of *bw//*R*;st//*R genotype was significantly higher than of *bw//*R*;st//st* genotype on both malathion concentrations (tested with an One-Way ANOVA after angular transformation). Thus, the third chromosome seems to harbour factor(s) that increase(s) resistance to malathion only when at least one second chromosome of the resistant strain is also present, indicating a significant genetic interaction between factors on the second and third chromosome.

Recessive autosomal factor(s)

The presence of recessive resistance factors (collectively denoted by 'r' in Figs. 5 and 6) was investigated using the dominant marker strain *Cy//Pm;Ser//Sb*. *Curly* and *Plum* are located on chromosome 2, whereas *Serrate* and *Stubble* are located on chromosome 3. Resistant females were crossed to males from the marker strain. F_1 males with the genotype *Pm//+;+//Sb* were backcrossed to resistant females, as shown in Fig. 5. Egg-to-adult survival of the F_2 flies from this cross was determined for different malathion concentrations and, after angular transformation, tested for significant differences with an One-Way ANOVA. In the absence of malathion, the four different genotypes from this cross are expected to be present in a 1:1:1:1 ratio.

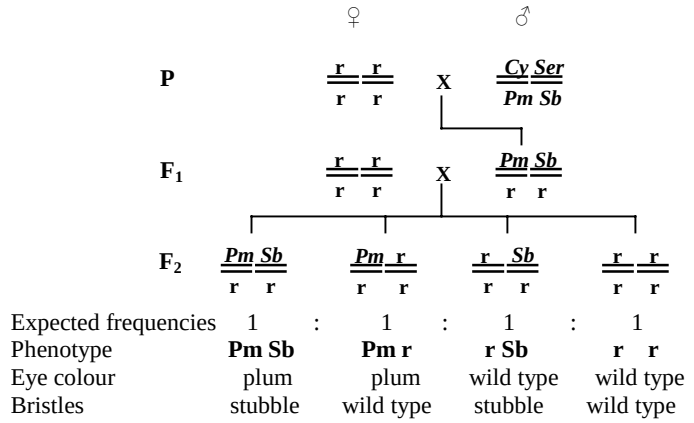

Figure 5. Crossing scheme to investigate the presence of recessive factor(s) on the autosomes by means of dominant markers. *Pm*=plum eyes (chromosome 2); *Sb*=stubbled bristles (chromosome 3). *Cy*=Curly wings (chromosome 2); *Ser*=Serrated wings (chromosome 3)

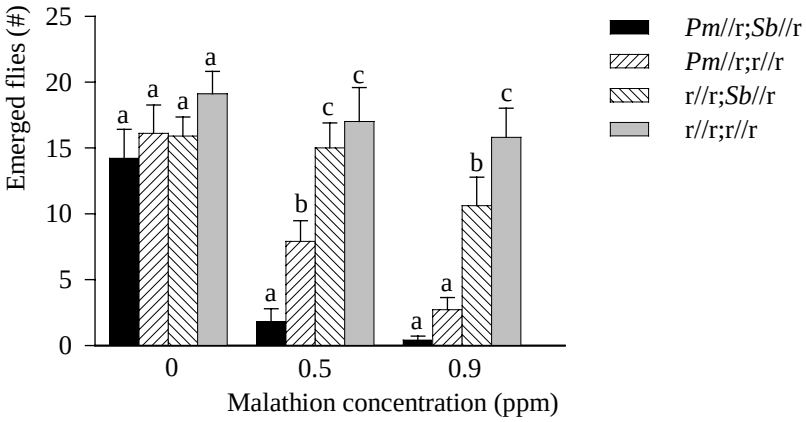

Figure 6. Mean egg-to-adult survival (+ s.e.) for different malathion concentrations. For each concentration, bars marked by the same letter indicate no significant difference in survival between genotypes for that concentration.

Figure 6 shows that on control medium the four genotypes are found in the expected ratio, as ANOVA revealed no significant differences (P=0.344). On malathion, the survival of the four genotypes was found to be significantly different (P<0.001) with *r//Pm;r//Sb* showing the lowest survival. Substituting the non-resistant marker chromosomes for both the second and third chromosome by resistant chromosomes increase survival, indicating that factors on both these chromosomes contribute to malathion resistance. The effect of the second chromosome seems to be stronger than that of the third chromosome, corroborating the observation that the second chromosome carries a factor improving resistance in heterozygous condition, as shown in the previous section.

General Conclusions

Several genetic factors contribute to malathion resistance in this *D. melanogaster* population, confirming earlier findings that malathion resistance is polygenically determined (Halpern & Morton, 1987; Houpt *et al.*, 1988). The X-chromosome, the second as well as the third chromosome contribute to malathion resistance, but clearly the second chromosome contributes

the most. This is in agreement with the localisation of several genes that have been reported previously to be involved in malathion resistance. Houpt *et al.* (1988) suggested that two cytochrome P450 genes (one on the second and one on the third chromosome) are involved in resistance. Also other studies suggest involvement of cytochrome P450's in malathion resistance (references in Wilson, 2001). Other genes associated with malathion resistance are glutathione-S-transferases, mapped both on the second and third chromosome (Morton, 1993), acetylcholinesterase, located on the third chromosome (Fournier *et al.*, 1989) and glucose-6-phosphate dehydrogenase on the X-chromosome (Morton & Holwerda, 1985). Further research mapping the factors involved in malathion resistance in this population in more detail, within the first, second and third chromosome, will clarify whether these 'candidate' genes are also responsible for the resistance observed in the present study.

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