SESSION 6D

FUNGICIDE AND INSECTICIDE RESISTANCE – CURRENT STATUS AND FUTURE MANAGEMENT

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SENSITIVITY OF WHEAT LEAF SPOT *SEPTORIA TRITICI* TO TEBUCONAZOLE

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ABSTRACT

Sensitivity of populations of *Septoria tritici* to tebuconazole from a total of 60 locations in France, Germany and the UK was studied in 1994 and 1995. Investigations based on more than 1500 isolates show that no evidence of selection for reduced sensitivity to tebuconazole in *Septoria tritici* could be found between 1994 and 1995. Field trials, complemented by glasshouse experiments, produced no evidence for a correlation between sensitivity of *S. tritici* populations and efficacy of DMIs. Essential factors influencing efficacy seem to be disease severity in relation to fungicide application timing.

INTRODUCTION

Leaf spot, caused by *Septoria tritici* is a common disease of wheat throughout the world. However, in the 1980 s, the occurrence of *S. tritici* in Europe was mostly restricted to coastal climates. Since the beginning of the nineties the economic importance of *S. tritici* has increased continuously and in the years 1992 to 1995, *S. tritici* was the dominant leaf spot disease of wheat in most parts of central Europe, locally attaining very high levels of disease incidence.

Demethylation-inhibiting fungicides (DMIs) are the most common fungicides used to control wheat leaf diseases and especially Septoria diseases. Generally, DMIs have proved very effective in practice and no complaints of reduced efficacy have been reported. However, special field trials under conditions of extremely high Septoria disease pressure, caused by using single treatments on very susceptible varieties, have shown that the performance of some DMIs (including tebuconazole) can sometimes disappoint under such extreme and atypical circumstances.

Given the fact that DMIs have been used against wheat diseases since the late 1970s, the hypothesis that a shift towards reduced sensitivity may have occurred needed to be examined. This paper reports the results of monitoring studies in 1994 and 1995 and the relationship between sensitivity and performance of fungicides against *S. tritici*.

MATERIALS AND METHODS

Monitoring field populations

Sensitivity tests in 1994 were carried out using a radial growth test in Petri dishes. Potato-dextrose agar plates were treated with 0, 0.03, 0.1, 0.3, 1, 3 and 10 µg a.i./ml tebuconazole. Colony diameter was measured 4 weeks after incubation and LC_{50} values were calculated from the diameters for each concentration.

However, with slow-growing fungi such as *S. tritici*, a radial growth test has the inherent methodological disadvantage of being very time-consuming and limited in precision, because of the very small absolute differences in the radial growth of different isolates.

Therefore, the 1995 monitoring was based on a new microtest adapted from a method described by Pijls *et al.* (1994). Thirty leaves infected with *Septoria tritici* were taken diagonally in the field from more than 40 locations in 1995 in western Europe (France, Germany and the UK). The sampling occurred from growth stage EC37 to EC85 in untreated or treated trial plots, or commercial cereal crops. Spores originating from a single pycnidium were scattered on Czapeck-Dox vegetable-juice agar. After 4 days of incubation, spores originating from a single colony were suspended in 3 ml of sterile glucose peptone medium (glucose 14.3 g/l; peptone 7.1 g/l) and incubated for 24 hours so that they had germinated.

Microtitre plates with 96 wells were prepared in the following way: each well of a row was first filled with 120 µl of a medium containing one of 8 different final concentrations (0, 0.003, 0.01, 0.03, 0.1, 0.3, 1 and 3 µg a.i./ml) of tebuconazole (or other azole fungicides), then 60 µl of a spore suspension were added to each of the fungicide solutions. A duplicate was prepared for each isolate, allowing 6 isolates to be tested per microtitre plate. The microtitre plates were then gently agitated at 20°C and 90% relative humidity for 6 days. Growth was determined with the aid of a microtitre plate reader (SLT, Spectra Image) using the absorbance at 405 nm, and LC_{50} values as well as statistical error were calculated according to the extinction values.

Glasshouse experiments

Five *S. tritici* isolates from 1995 (representing LC_{50} values from 0.05 to 1.3 ppm tebuconazole) were selected for this study. The wheat cultivars cv.Goupil, cv. Sidéral, cv.Forby and cv.Soissons, having different susceptibilities to *S. tritici* were used. Seedlings were inoculated at the two-leaf growth stage and treatments with tebuconazole or metconazole were applied 3 days after inoculation at the rate of 500 ppm a.i.. (five replications). The disease severity in terms of % necrosis was assessed after 21 days incubation at 20°C. In addition, 28 days after inoculation, the number of pycnidia was counted on 5 leaves. These data were used to evaluate the efficacy of tebuconazole and metconazole.

RESULTS AND DISCUSSION

Sensitivity Spectrum

A total of more than 1500 isolates of *S. tritici* from the three countries were tested in 1994 and 1995. Figure 1 illustrates the sensitivity profile to tebuconazole of *S. tritici* populations in Europe for both years.

Because the test method was changed between 1994 and 1995, no direct comparison of sensitivity profiles was possible for these two years. However, numerous 1994 isolates with LC_{50} values belonging to the different sensitivity classes were tested in the following year with the microtest method. The results demonstrated that, in general, the sensitivity ranking remained essentially unchanged between 1994 and 1995 (0.01 to 1 ppm). Furthermore, absolute values measured with the aid of the microtest were generally lower (Table 1).
Only those isolates showing high LC_{50} values in the radial growth test (> 1 ppm) turned out to be different in the microtest. It could be demonstrated that most of the isolates showing

high LC_{50} values were slow-growing, and with this type of isolate, due to the very small absolute differences in radial growth at different concentrations, the exactness of measuring the colony diameter appears to become a limiting factor.

LC_{50} values obtained in 1994 and 1995 show a 100-fold difference between the most sensitive isolate (0.01 ppm) and the least sensitive isolate (1.2 ppm). Similar results were obtained by Hermann and Gisi (1994) with cyproconazole and flutriafol. However, more than 85% of the isolates show LC_{50} values between 0.03 and 1 ppm, which represents a factor of 30.

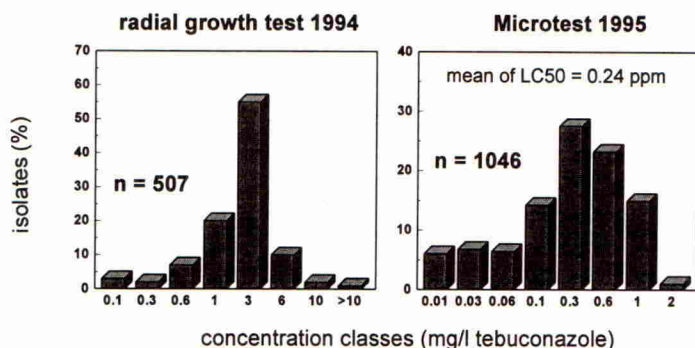Figure 1. Distribution of LC_{50} values in France, Germany and the UK.

concentration classes (mg/l tebuconazole)

Table 1. LC_{50} value of isolates obtained in 1994 and tested with radial growth test in 1994 and with the microtest in 1995.

| isolate	1994 - radial growth test LC_{50} values (ppm tebuconazole)	1995 - microtest LC_{50} values (ppm tebuconazole)
449	0.05	0.01
386	0.07	0.01
507	0.08	0.03
275	1.4	1
143	1.4	0.4
312	6.9	0.4
27	8.8	0.6
43	>10	0.7

Regional distribution and variety influence

Figure 2 shows the distribution of sensitivities in different regions of France in 1995.

Although the number of investigated isolates was not identical in the different regions, no significant differences could be observed within the regions in the northern and central part of France.

The sensitivity patterns of *S. tritici* populations isolated from different cultivars (cv. Goupil, cv. Soissons and cv. Forby) grown on one site were also investigated. Results showed that disease attack varied considerably according to the cultivar; cv. Goupil developed a high level of disease, whereas cvs. Forby and Soissons suffered only a low attack. Although the cultivars showed very different susceptibility levels to *S. tritici*, sensitivity patterns were

similar for cultivars grown at this one site. Consequently, cultivar does not seem to play a role in the selection of *S. tritici* populations in terms of sensitivity.

Figure 2. Regional distribution of LC_{50} values (ppm tebuconazole) in northern and central France.
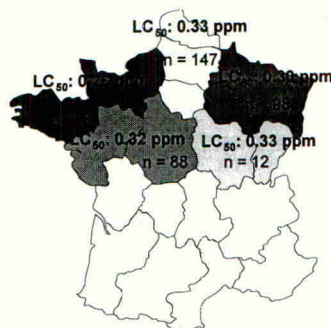
Field performance

Figure 3. Relationship between sensitivity, disease pressure and field performance
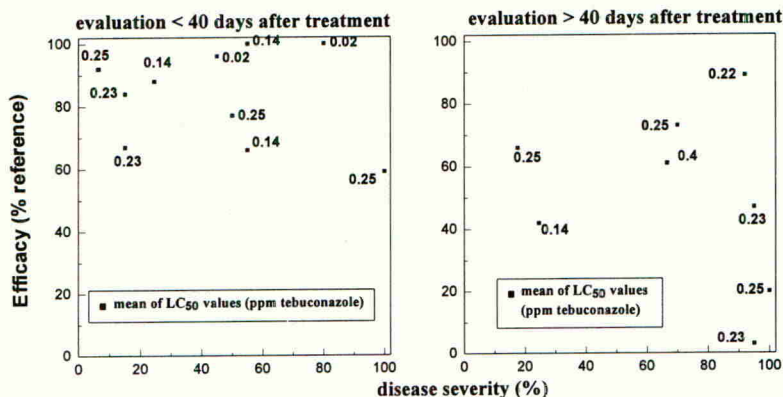
Using to data from 10 field trials, the relationship between sensitivity level (mean of LC_{50} values), disease pressure (percentage disease severity) and field performance of tebuconazole (as Folicur 250EW) were investigated (Figure 3); field performance relative to epoxiconazole was assessed up to, and later than, 40 days after treatment.

Results showed no direct correlation between sensitivity, disease pressure and field performance when assessment was made within 40 days after treatment. Assessments later than 40 days after treament showed that under very high levels of attack (>90%), the persistent effect of tebuconazole may be reduced significantly, independently of the sensitivity.

At similar levels of attack and sensitivity, the timing of the fungicide application determined the efficacy level. When conditions of very high disease pressure were combined with a non-optimal fungicide timing, efficacy of tebuconazole could be reduced.

Glasshouse trials

Figure 4 shows the efficacy of tebuconazole and metconazole as mean values for the four cultivars. The efficacy was determined according to either the extent of leaf necrosis or the number of pycnidia.

Results based on % necrosis showed an efficacy of 50 to 70% for tebuconazole and 65 to 75% for metconazole. Assessment of efficacy according to number of pycnidia showed excellent disease control with both fungicides.
Observations established by the comparison of sensitivity and field performance were confirmed. In glasshouse experiments on cereal plants, no correlation could be found between sensitivity of the isolates investigated and fungicide efficacy.

Figure 4. Efficacy of tebuconazole and metconazole under greenhouse conditions
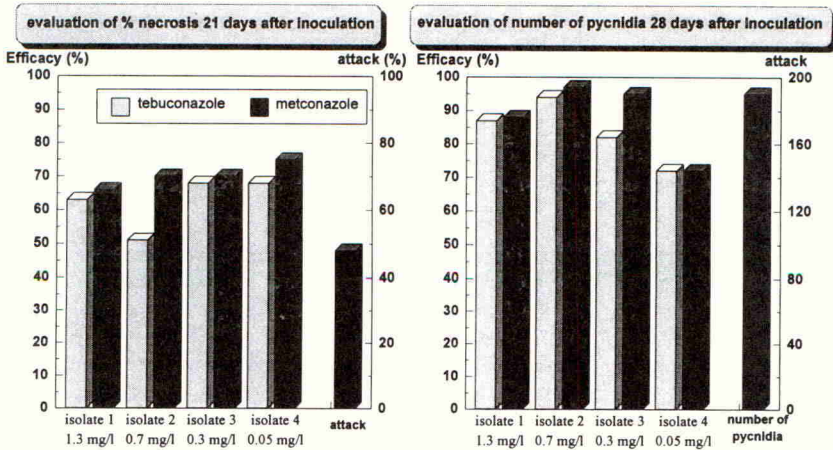
Cross-Sensitivity Studies

Figure 5. Cross-sensitivity patterns between tebuconazole and other triazoles (LC_{50} values in ppm a.i)
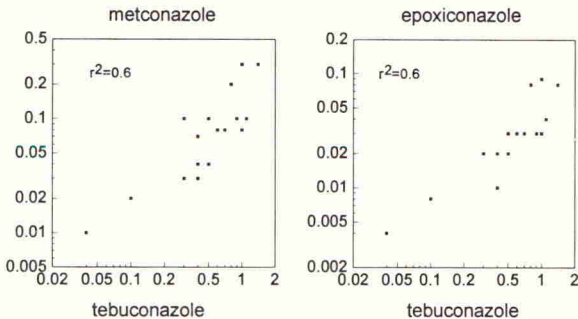
Cross-sensitivity-studies carried out with tebuconazole, metconazole and epoxiconazole showed clearly a positive cross-sensitivity betweeen these three active ingredients (Figure 5). These results are in accordance with those of numerous studies concerning DMIs and other fungal pathogens, reported earlier (Hermann and Gisi , 1994; Anon., 1995; 1996).

CONCLUSION

Favourable climatic conditions in 1994 and 1995 for the development of wheat leaf spot *S. tritici* resulted in high disease pressure throughout the season.

The results of intensive sensitivity monitoring carried out in 1994 and 1995 in western Europe showed that there is no evidence of a shift to less sensitive isolates over the two years. Disappointing results in some field trials were more likely caused by extreme conditions such as very high disease pressure combined with non-optimal application timing of fungicide. Field and glasshouse experiments showed clearly that at present no correlation could be found between the sensitivity of *S. tritici* populations and performance of products containing tebuconazole.

From the data presented here, it seems to be rather unlikely that a significant change in the sensitivity of *S. tritici* populations in France, Germany and UK towards DMIs, and in particular tebuconazole, has occurred from 1994 to 1995. This is confirmed by other workers (Anon., 1995,1996) who were also unable to detect any significant changes. Nevertheless, in view of the intensive use of DMIs in cereals monitoring should be continued.

ACKNOWLEDGMENTS

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MONITORING TRIAZOLE FUNGICIDE SENSITIVITY IN POPULATIONS OF *SEPTORIA TRITICI*

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ABSTRACT

Isolates of *Septoria tritici* collected annually from field crops located throughout England and Wales during 1992, 1994 and 1995 were tested for the degree of sensitivity to prochloraz, propiconazole, triadimenol, flutriafol, tebuconazole and cyproconazole at a range of concentrations *in vitro*. Preliminary analysis indicated that propiconazole and prochloraz were the most effective fungicides against isolates of *S. tritici in vitro*. Triadimenol was the least effective whilst tebuconazole, cyproconazole and flutriafol were intermediate in their effectiveness. For populations tested, there appears to have been a shift towards a reduction in sensitivity to triadimenol since 1992. A shift in sensitivity was also recorded for flutriafol but to a lesser extent than for triadimenol. Reactions to propiconazole and prochloraz were relatively consistent, although results in 1995 showed possible progress towards a reduction in sensitivity. Populations showed consistent responses to tebuconazole and cyproconazole in 1994 and 1995. Overall, isolates in 1995 showed a greater diversity in sensitivity and therefore a higher potential for growth in the presence of fungicide than in previous years. Possible implications of these results are discussed.

INTRODUCTION

Septoria tritici, the cause of leaf blotch, is the most important pathogen of winter wheat in the UK. Annual losses due to this disease have been estimated to be in the range of £4.3- 87M depending on disease severity in individual years. All wheat crops are potentially at risk from this disease and severe attacks may occur frequently but particularly on very susceptible cultivars. Fungicide application is the only means of achieving a high degree of disease control since most, if not all, current commercial cultivars are susceptible to a greater or lesser degree. Inevitably this leads to fungicide application in 95% or more of wheat crops with *S. tritici* as the primary target disease.

Since the introduction of triazole fungicides to the UK in 1984/85 the percentage of crops that receive one or more applications of a triazole fungicide has risen from zero to almost the entire crop. These demethylation inhibitor (DMI) fungicides, which include cyproconazole, tebuconazole, prochloraz (an imidazole fungicide), propiconazole, flutriafol and triadimenol, now dominate fungicide use on winter wheat. The widespread use of DMI fungicides may therefore lead to considerable and consistent (season-to-season) selection pressure being exerted on populations of *S. tritici* and increase the likelihood of populations becoming less sensitive to triazole fungicides.

Monitoring sensitivity to these fungicides has an important role in the formulation of anti-resistance strategies. Results of a Home-Grown Cereals Authority (H-GCA) funded survey of DMI sensitivity in populations of *S. tritici* have established base-line responses *in vitro* to four fungicides commonly used for control of the disease (Hims, 1993). Results also confirmed reduced sensitivity to flutriafol or triadimenol in some populations. This paper reports further results of *in vitro* monitoring of sensitivity to DMI fungicides carried out between 1992 and 1995.

MATERIALS AND METHODS

Spore production and isolation

Wheat leaves showing symptoms of *Septoria tritici* infection were selected from plants sampled from a range of different cultivars collected as part of the MAFF national winter wheat disease survey at the early- to medium-milk growth stage (GS 73-75). Selected lesions were surface sterilised for 10-20 seconds in a 1% solution of sodium hypochlorite, rinsed in sterile water and dried with sterile paper tissue. Leaf sections were placed in Petri dishes on tap water agar and incubated at 17°C under near u.v. light for 24 hours. From each leaf a random cirrhus was picked off and streaked onto potato dextrose agar amended with streptomycin. Plates were incubated under near u.v. light at 17°C for 7 days.

After 7 days plates were washed with 10ml sterile water and the resulting spore suspension filtered through sterile tissue into a sterile universal bottle. All spore suspensions were adjusted to a concentration of 1.0×10^8 spores per ml and stored at 5°C.

Determination of EC_{50} values

At the beginning of each year of the project a selected number of isolates were tested against a wide range of concentrations in order to determine a dose-response curve for each fungicide. In 1995, six fungicides: flutriafol, triadimenol, propiconazole, prochloraz, cyproconazole and tebuconazole were tested against sixteen isolates with three replicates used per isolate. Molten agar at 50°C was amended with fungicide stock solutions, mixed thoroughly, and poured into Petri dishes. The range of final fungicide concentrations was 0; 0.1; 0.178; 0.316; 0.56; 1.0; 1.78; 3.16; 6.3 mg/l a.i. Plates were streaked with spore suspension and incubated at 17°C in the dark for 8 days. Growth of each isolate was assessed on amended plates after comparison with growth of the isolate on unamended agar. Isolate growth was graded on a growth category scale from 0 to 10 where 10 indicated growth equivalent to the control and 0 indicated complete inhibition. A dose-response curve was fitted to the data and the EC_{50} value, the concentration reducing growth of isolates by one half, for each fungicide estimated.

For comparison, an alternative method of measurement of fungicide sensitivity based on the use of light absorbance to measure fungal growth (Pijls *et al.*, 1994) was also used to determine EC_{50} values for the six fungicides tested.

Screening of isolates

Approximately 100 isolates originating from crops throughout England and Wales were screened each year against two concentrations of each fungicide. All isolates were tested for sensitivity at the individual EC_{50} for each fungicide and at an additional concentration representing a rate of ten times the EC_{50}. Growth in the absence of fungicide was measured on unamended agar plates. Plates were prepared and assessed as described above. Data presented show results from screening at the EC_{50} value for each fungicide.

RESULTS

Results show that propiconazole and prochloraz were the most effective fungicides tested against populations of *S. tritici in vitro* (Figure 1). Triadimenol was the least effective fungicide with some isolates showing uninhibited growth at concentrations above 10 mg/l a.i. Tebuconazole, cyproconazole and flutriafol were intermediate in their effectiveness. With the exception of triadimenol, EC_{50} values calculated annually for each of the fungicides did not significantly alter during the period of testing. In 1991, the EC_{50} for triadimenol was measured at 3 mg/l a.i. but in 1995 the value had increased to 5 mg/l. For the purposes of comparison between years, the value of 10 mg/l a.i. was used as an additional concentration to screen isolates for sensitivity to triadimenol. The alternative method using light absorbance to measure growth produced estimations of EC_{50} values which were very similar to those obtained using standard agar techniques.

Annual *in vitro* testing indicated there was a shift towards a reduction in sensitivity to triadimenol in the populations of *S. tritici* tested (Figure 2). Comparison of results from national monitoring in 1992 and 1995 show a shift in the population with the majority of isolates in 1995 showing less than 50% inhibition at a concentration of 10 mg/l a.i., a concentration which in 1991 had completely inhibited growth of over 35% of the isolates tested. For flutriafol, the shift was less pronounced although at 0.5 mg/l over half the isolates tested in 1995 showed less than 10% inhibition of growth compared to the unamended controls (Figure 3). Reaction to propiconazole indicated what appears to be a transitional position in the possible progress towards reduced sensitivity. Isolates in 1995 showed more diversity in sensitivity and therefore a higher potential for growth in the presence of the fungicide than in previous years (Figure 4). Populations continued to react very similarly to prochloraz during the three years of testing although there was a tendency towards reduced sensitivity in 1995 (Figure 5). Tebuconazole and cyproconazole both exerted a similar degree of effect on the growth of isolates *in vitro* (Figures 6 & 7). Populations showed similar growth distributions in response to both tebuconazole and cyproconazole in 1994 and 1995 but growth distributions for isolates on agar amended with tebuconazole showed a bi-modal pattern which may indicate the existence of two different populations (Figure 7).

DISCUSSION

Evidently there were indications from *in vitro* tests of the development of reduced sensitivity to triadimenol, illustrated not only by a shift in sensitivity in the population but also a two-fold

Figure 1. *In vitro* dose response curves for fungicides tested (1995)
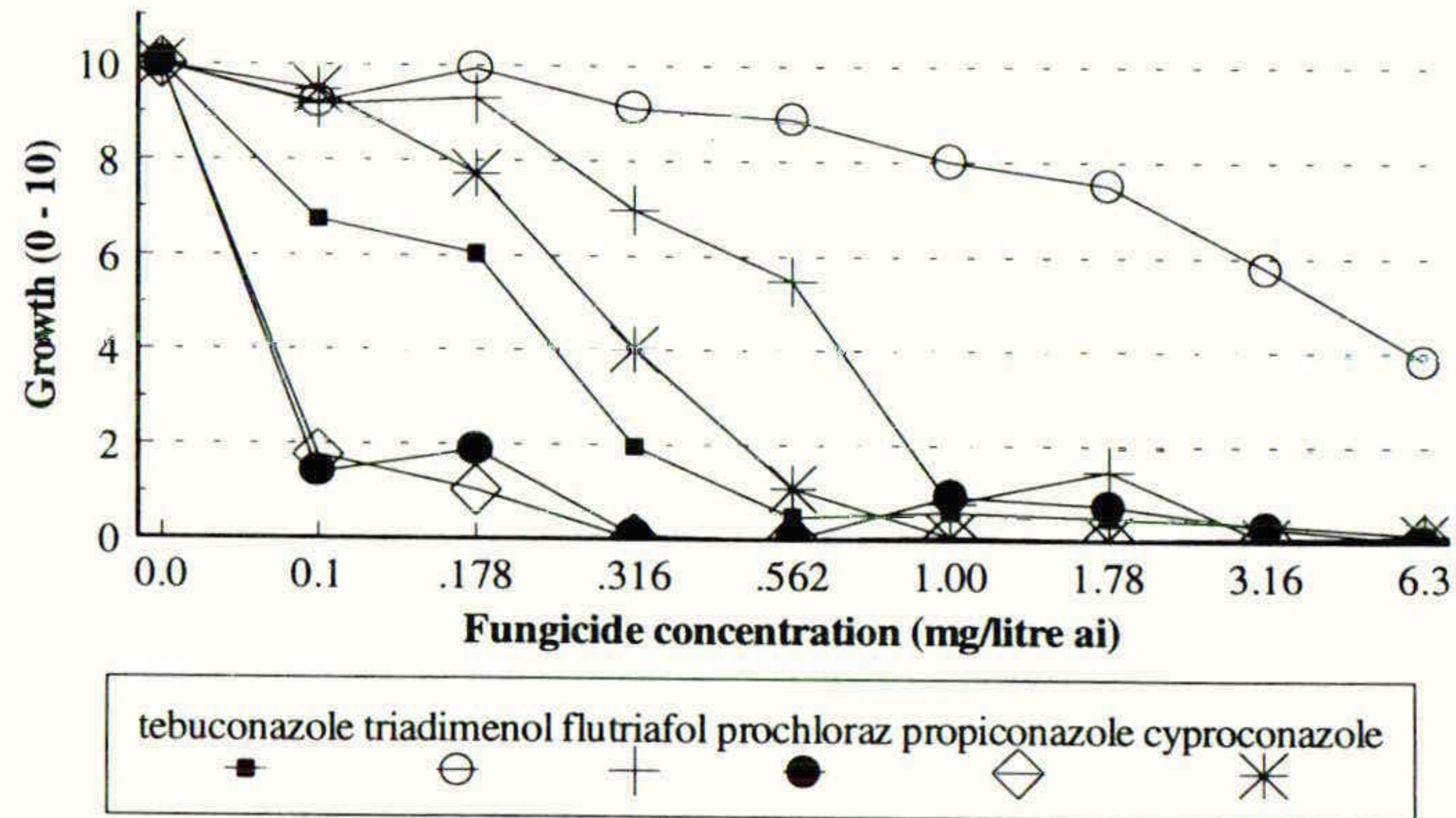
Sensitivity of populations of *S. tritici* to selected fungicides (1992-1995)

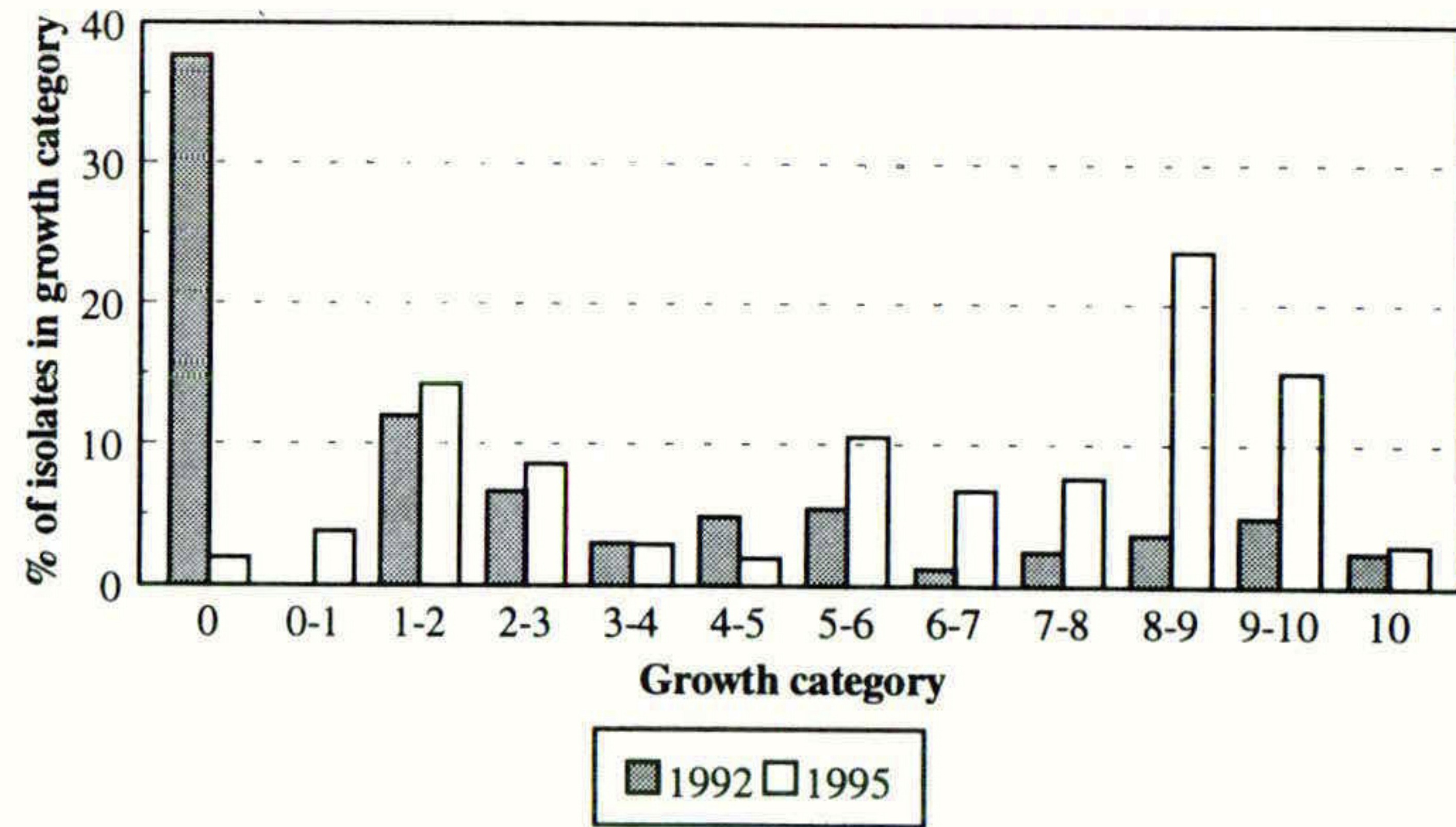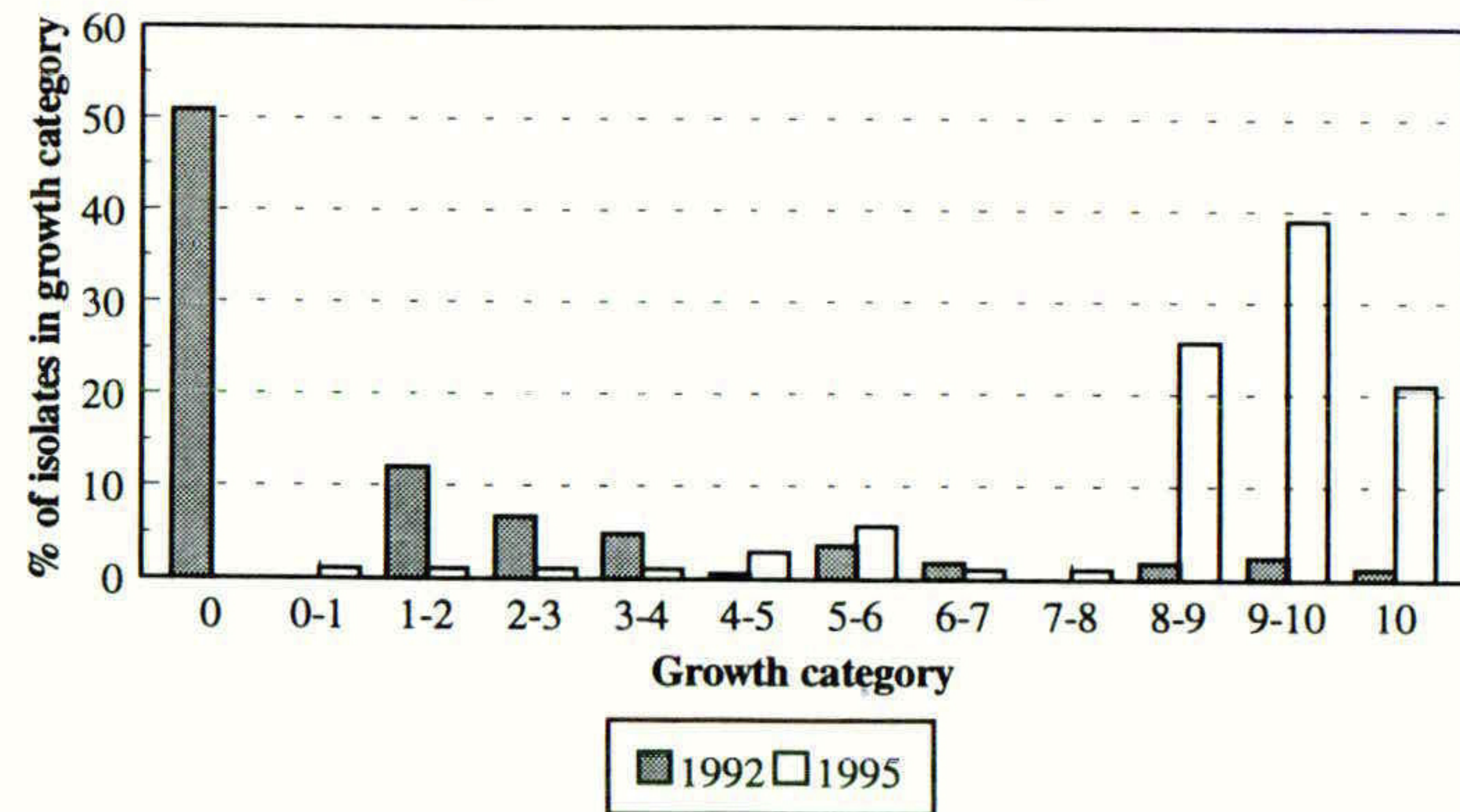Figure 2. Triadimenol - 10 mg/l ai

Figure 3. Flutriafol - 0.5 mg/l ai

Sensitivity of populations of *S. tritici* to selected fungicides (1992-1995)

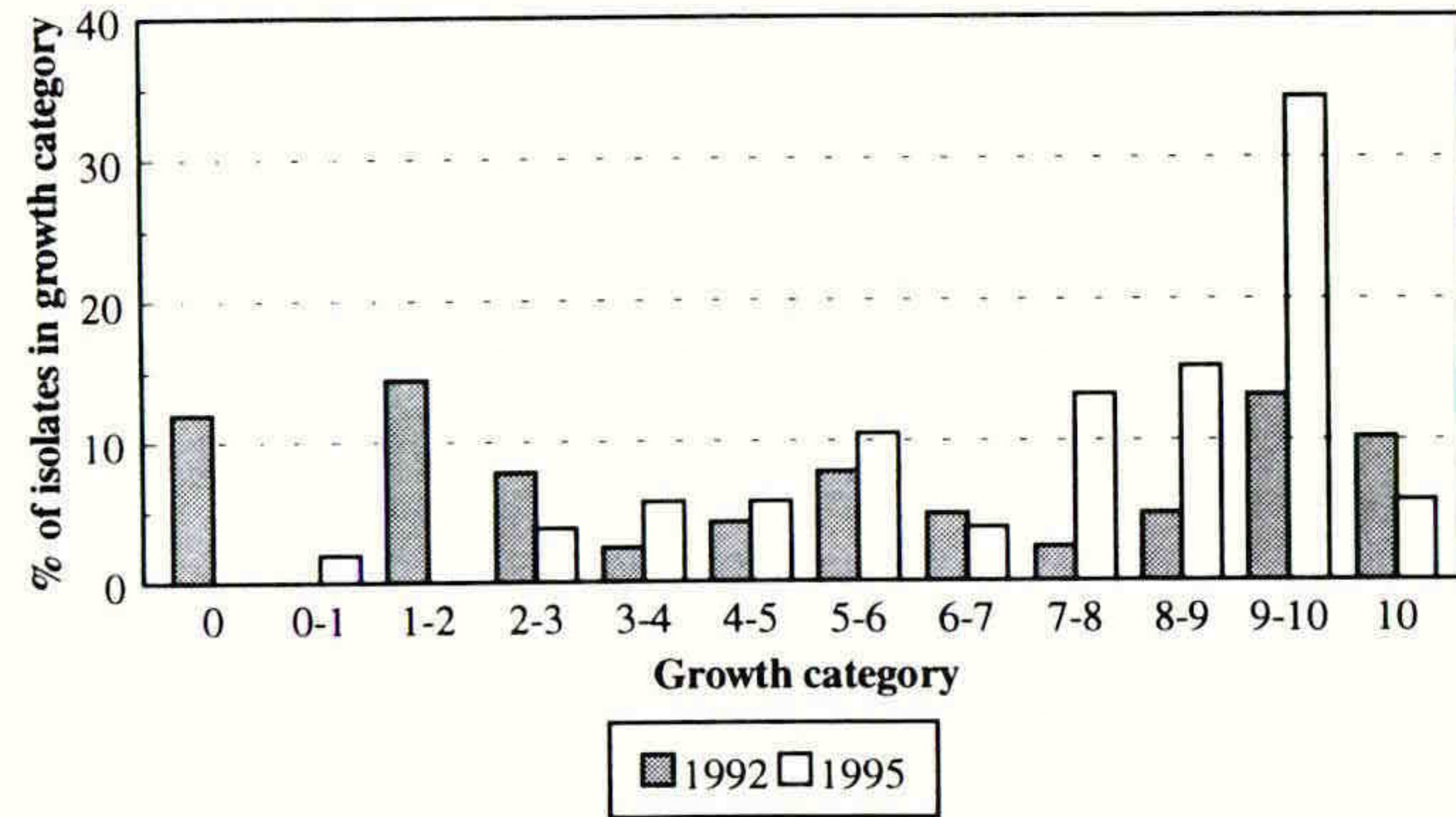Figure 4. Propiconazole - 0.05 mg/l ai
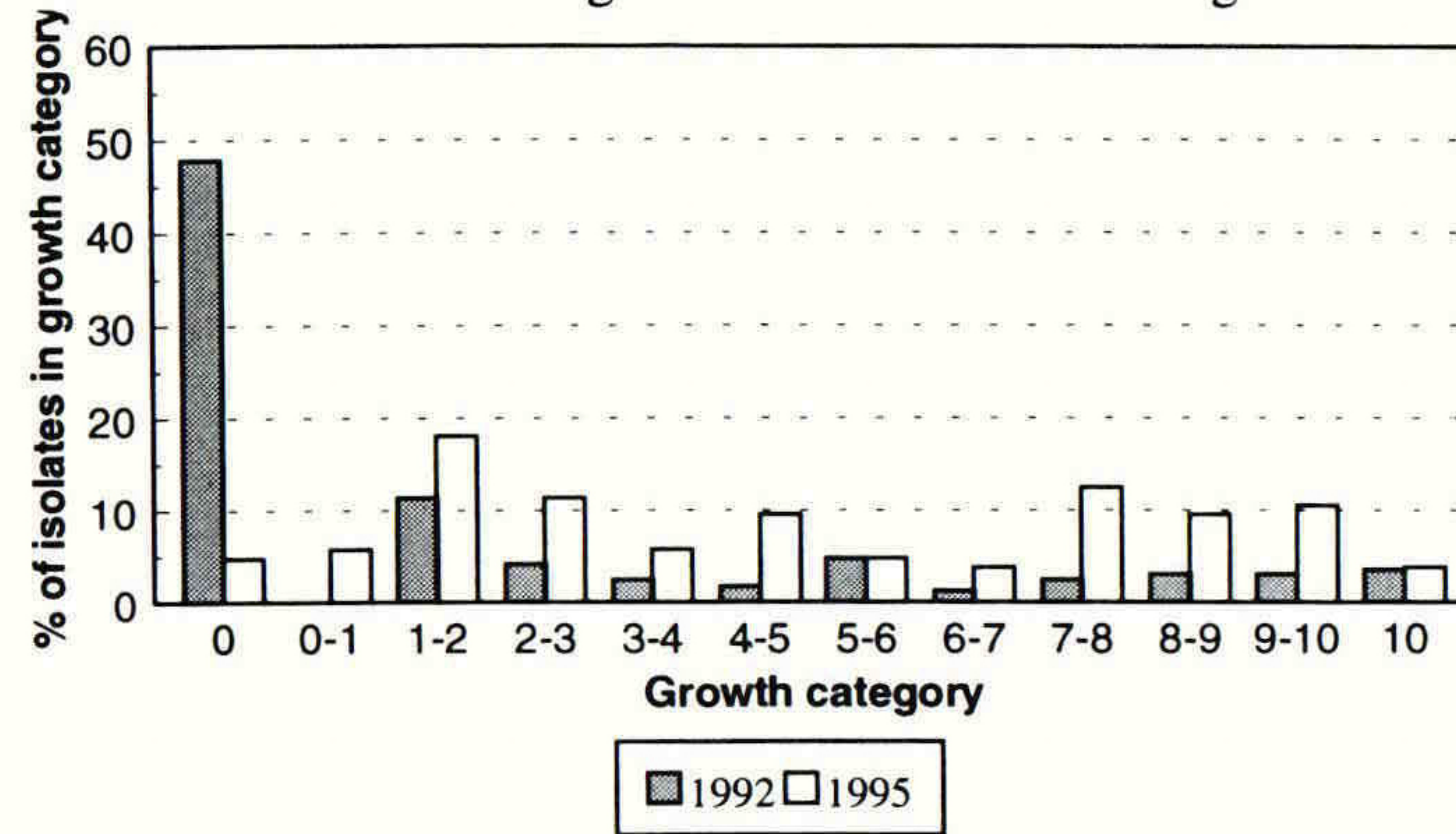

Figure 5. Prochloraz - 0.05 mg/l ai
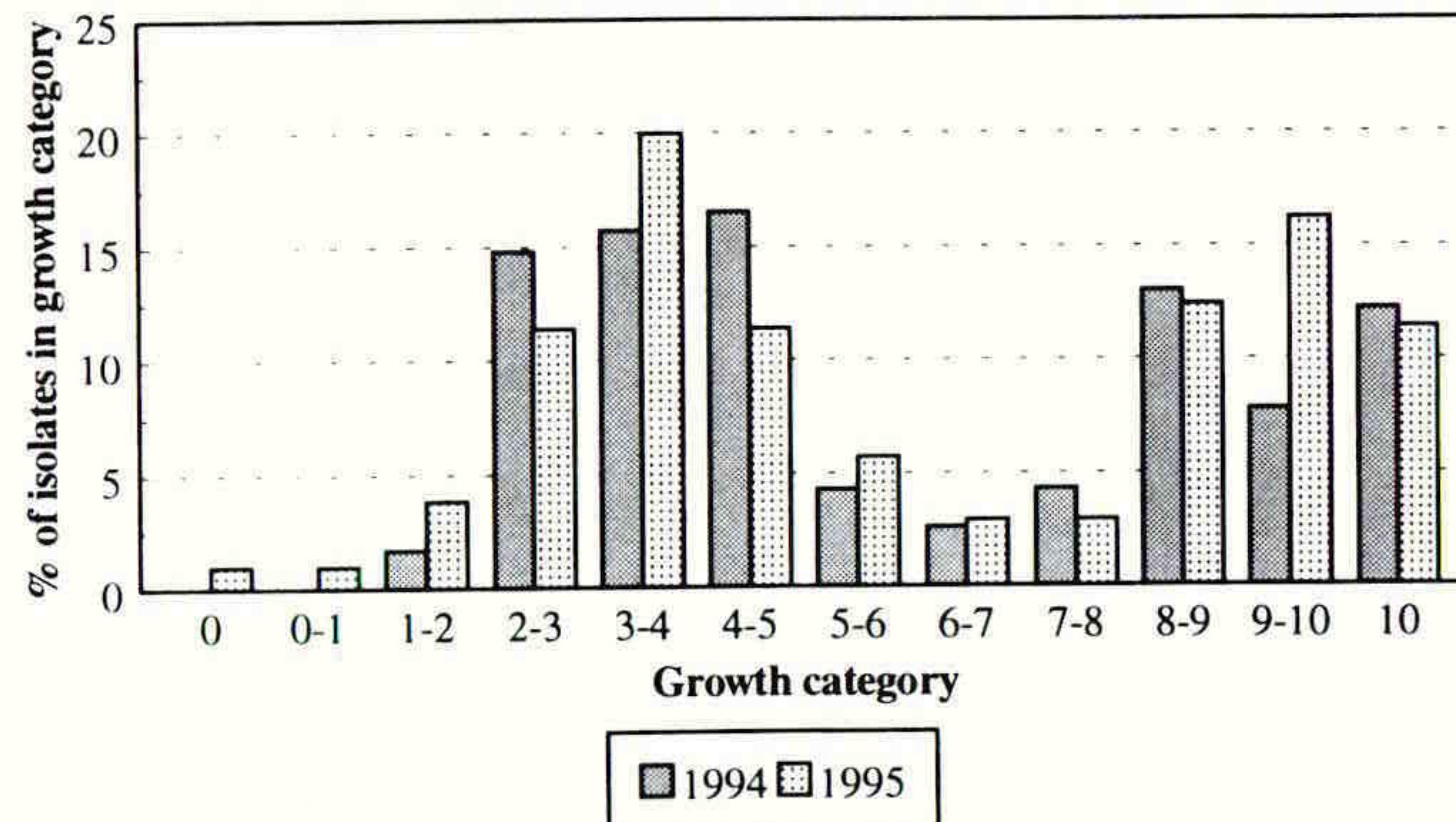

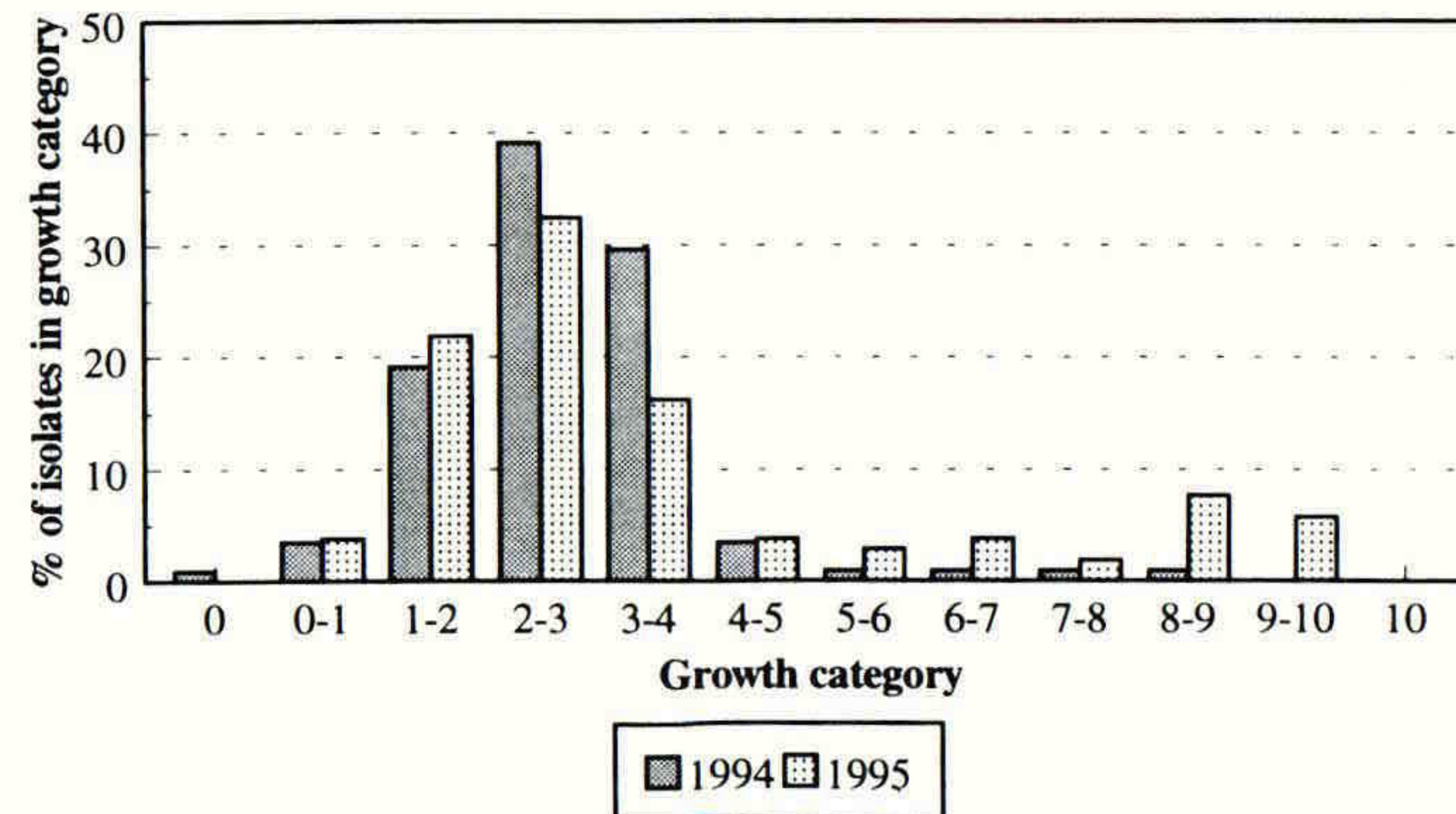Figure 6. Tebuconazole - 0.2 mg/l ai

Figure 7. Cyproconazole - 0.3 mg/l ai

increase in the EC_{50}. In addition, a less pronounced shift in sensitivity to flutriafol was indicated. The explanation for these shifts, not seen in prochloraz and propiconazole, which have been used as extensively as triadimenol and flutriafol, is unclear. However, the occurrence of a shift in sensitivity in the *S. tritici* population to a DMI group fungicide highlights the need for vigilance in monitoring, especially as the newer generation triazoles are being widely used at reduced rates with a higher frequency of application than the older DMIs. Data from the MAFF Pesticide Usage Survey 1994 (Garthwaite *et al.*, 1994) show that of the triazole fungicides applied to wheat, tebuconazole was the most commonly applied and was also the fungicide most often used at reduced rates. Twenty percent of applications of tebuconazole were made at 25% recommended rate and 30% of sprays of cyproconazole were applied at 50% recommended rate (Thomas, personal communication). Reduced rates may not be so effective and in the presence of isolates/populations with reduced sensitivity, failure to control disease could become a distinct possibility. The degree of selection pressure created by repeated low dose applications could lead to a shift in the population towards reduced sensitivity to these fungicides.

It is unclear at this stage whether the shift in sensitivity recorded to specific DMI fungicides is seasonal or if it will progressively reduce the overall sensitivity of *Septoria tritici* to triazole fungicides. The bi-modal distribution of sensitivity of populations to tebuconazole could indicate the existence of two different populations and may denote a potential for reduced sensitivity to this fungicide. Clearly use of strategies such as co-formulation or tank mixing of DMI fungicides with a protectant fungicide such as chlorothalonil or another fungicide are extremely important in the maintenance of the efficacy of the DMI fungicide group.

Further monitoring will be undertaken during 1996. Additionally, isolates from each year will be tested concurrently to quantify between-year variability in results. Field experiments have been initiated to determine whether isolates showing reduced sensitivity to DMI fungicides in the laboratory are less effectively controlled in the field. Preliminary results indicate that this may be so.

ACKNOWLEDGEMENTS

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RESISTANCE PROFILING OF THE NEW POWDERY MILDEW FUNGICIDE QUINOXYFEN (DE-795), IN CEREALS

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ABSTRACT

Quinoxyfen (DE-795) is a powdery mildew specific fungicide from a new chemical class for use in cereals, grapes and vegetables. A resistance profiling programme has been carried out to understand the characteristics of quinoxyfen and to allow a resistance management programme to be initiated. This consisted of cross-resistance studies, baseline monitoring and determining the fitness of mutagenised mildew. The baseline sensitivity of 340 field isolates, collected during 1991-1994, of *Erysiphe graminis* f.sp. *hordei* to quinoxyfen ranged from ED_{50} values of <0.0001-0.16 mg/litre, with a mean of 0.003 mg/litre. No evidence of cross-resistance between the azole, triadimenol, and quinoxyfen was found. Quinoxyfen 'resistant' strains of *E. graminis* f.sp. *hordei* generated in the laboratory by mutagenesis, or collected from a treated field crop, were of low fitness and required quinoxyfen for their survival.

INTRODUCTION

The development of resistance to fungicides by *Erysiphe* spp. of cereals has been well documented for ethirimol (Hollomon, 1975) and sterol demethylation inhibitors (DMIs) by (DeWaard, 1994). It is also a requirement for registration of new plant protection products under EC legislation that an assessment of resistance risk, and a resistance management statement be supplied (Anon., 1991 and Anon., 1993). As part of the development of quinoxyfen (DE-795), a mobile fungicide which controls powdery mildew of cereals by preventing infection (Longhurst *et al.*, 1996), the cross-resistance of quinoxyfen to current cereal fungicides, the baseline sensitivity profile, and fitness of mutants was assessed.

MATERIALS AND METHODS

Cross-resistance

The bioassay method described in Hollomon (1982) was used. Briefly, leaf segments of barley (cv. Halcyon) were floated on solutions of fungicide. Concentrations of quinoxyfen usually ranged from 0.0005 to 0.05 mg/litre. Inoculation of the segments with selected mildew isolates (using a settling tower) and their subsequent incubation and examination under a microscope enable measurements of germ tube growth and inhibition of appressorium formation to be made. Infection and growth were measured after 72 hours incubation at 15°C, and sensitivity determined as the dose needed to reduce growth by 50% (ED_{50}).

Baseline sensitivity monitoring

In 1991, samples were collected from untreated winter barley sites throughout England and Scotland. From 1992 - 1994 sampling was confined to a 35 x 50 metre block of winter barley (cv. Pastoral) which was sprayed with 250 g/ha of a suspension concentrate formulation of quinoxyfen at second node growth stage (BBCH32-Bleiholder *et al.*, 1989).

Plants (cv. Golden Promise) were grown, 5 plants per pot, in a closed greenhouse and treated when three leaves were fully expanded with 250 g/ha of formulation of quinoxyfen. An equal number of plants were left unsprayed. One pot of treated plants and one pot of untreated plants were placed in a shallow aluminium tray lined with capillary matting. Sampling of the mildew population was carried out by placing these pairs of pots randomly through the treated crop. The treated and untreated sampling pots were placed in the crop at 21 and 42 days after treatment of the crop and collected one week later. The bait plants were incubated for a week in a polythene tunnel before single pustule isolates were made. Leaf segments containing single pustules were cut from bait plants and allowed to sporulate. These single pustule clones were transferred to fungicide-free leaf segments and maintained at 15°C and subcultured at 10-day intervals.

Colonies collected in 1991 and 1992 were transferred to untreated plants for maintenance before assay. After the discovery through mutagenesis experiments in 1992 that any mutants created were of low pathogenicity and dependent on quinoxyfen for viability, material was also maintained on treated plants. The bioassay methods are as described in Material and Methods: Cross-resistance.

Mutagenesis

Ten-day old barley seedlings (cv. Halycon) were inoculated with conidia of a wild-type barley mildew isolate (23D5), which had never before been exposed to fungicides. After 24 hours the first leaves were removed and their cut ends placed in 2.0 ml of a solution containing 2-aminobenzimidazole (100 mg/litre) and N-nitrosoguanidine (NTG; 25 mg/litre). Uptake was allowed to continue for four days and the segments were then transferred to water agar (0.5% w/v) for sporulation to occur. Clones were established on fungicide-free leaf material from both the NTG-treated and untreated sources. The number

Table 1. Cross-resistance patterns in barley powdery mildew to quinoxyfen and triadimenol. (Library isolates)

Strain	Year of Isolation	Sensitivity (ED$_{50}$ mg/litre)	
		Triadimenol	Quinoxyfen
DH14(1)	1976	0.004	0.001
DH14(2)	1976	0.008	0.008
23D5	1973	0.007	0.06
JB212	1982	0.459	0.0005
CSB5	1989	0.51	0.009
CSB22	1989	0.62	0.002
BSB18	1989	0.70	0.081
CSB9	1989	0.70	0.003
JB1753	1988	1.68	0.005
CSB2	1989	1.72	>5.0
JB1931	1989	1.85	0.071
JB1641 (1)	1987	2.06	0.004
JB1641 (2)	1987	7.8	0.015
BUSB22	1989	4.3	0.008
BUSB6	1989	4.8	0.1
BUSB20	1989	6.8	<0.0005
L32	1989	6.0	0.0008
BUSB2	1989	7.4	0.09
1704*	1987	1.78	0.01
1705*	1988	0.72	0.0008
E10*	1986	2.24	0.002

Using this laboratory test, differences must be greater than 10-fold to achieve significance at the 95% level (validated for triadimenol). Tests carried out in 1990. * Tests carried out in 1991.

Table 2. Distribution of quinoxyfen sensitivity in barley powdery mildew. Composite data for all isolates 1991-1994.

ED$_{50}$ (µg/ml)	Number of isolates							
	1991		1992		1993		1994	
	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Total
<0.0001-0.0005	3	1	2	3	0	1	0	10
0.0005-0.001	12	7	19	7	10	4	6	65
0.001-0.005	9	8	35	12	12	11	12	99
0.005-0.01	5	9	29	5	25	6	10	89
0.01-0.05	10	5	18	9	5	0	4	51
0.05-0.1	2	2	8	4	1	1	2	20
0.1-0.5	2	1	0	1	1	0	1	6
0.5->2	0	0	0	0	0	0	0	0
Total	43	33	111	41	54	23	35	340

Mutagenesis

Exposure of the wild-type isolate 23D5 to NTG, followed by selection on quinoxyfen-treated leaves generated mutants which were able to grow on these treated leaves.

Bioassay of five of these mutants showed an ED$_{50}$ value in excess of 0.5 mg/litre,

of pustules that developed from both inoculated sources provided a measure of the % kill caused by N-nitrosoguanidine.

These clones were transferred with a paint brush to leaves of the same barley cultivar sprayed to run-off with a suspension concentrate formulation of quinoxyfen (0.16 mg/litre). Growth was assessed after 7 days.

Conidia production

Seedlings infected with either IW 240 ('resistant' isolate, collected from the field in 1993) or 23D5 (wild-type) were shaken after 7 days to remove existing conidia. Forty-eight hours later, ten leaves each with, as far as possible, similar levels of infection, were carefully removed and placed in 2.5 ml of 50% ethanol. Tubes were shaken and conidia counted using a haemocytometer. Conidia production was expressed as the number produced per leaf.

Conidia produced after exposure to NTG for 4 days were inoculated onto quinoxyfen-treated or untreated leaf segments, and pustule numbers counted 7 days later. This infection provided the inoculum source for the same testing procedure up to the fourth generation.

RESULTS AND DISCUSSION

Cross-resistance

No cross-resistance between triadimenol and quinoxyfen was observed (Table 1). Isolate DH14 is ethirimol resistant (Hollomon, 1982) suggesting no cross-resistance between quinoxyfen and this 2-aminopyrimidine mildewicide. With one exception (CSB2), all the 'library' isolates were quinoxyfen sensitive with ED_{50} values varying from >0.0005 to 0.1 mg/litre. Unfortunately, no further work was carried out on CSB2 as it was soon lost from the culture collection. In this respect, CSB2 resembled mutants subsequently produced in the laboratory by mutagenesis with NTG (e.g. NTG1), or collected from the field in quinoxyfen-treated crops. Nevertheless, the presence of CSB2 led to the setting up of a baseline survey of field collected isolates.

Baseline Monitoring

The overall sensitivity distribution of isolates to quinoxyfen is shown in Table 2. The median peak is in the 0.001-0.005 mg/litre range (0.003 by measurement of the median of the concentration range). There was no significant difference between isolates collected from the field on either treated or untreated plants.

emphasising the difference from the parent wild-type isolate (0.06 mg/litre; Table 1). All these mutants seemed to require the presence of quinoxyfen for normal infection and growth; in the absence of the fungicide, mutants grew poorly and could not be maintained on untreated leaves (Table 3).

Table 3. Viability of wild type (23D5) and laboratory mutant (NTG1) in the presence or absence of quinoxyfen (0.16 mg/litre).

	Fungicide treatment	Number of pustules/leaf after each generation			
		1	2	3	4
Wild-type	No quinoxyfen	115	84	73	127
(23D5)	+ quinoxyfen	4	0	0	0
NTG1	No quinoxyfen	17	6	8	1
	+ quinoxyfen	63	85	98	82

Table 4 shows the number of conidia produced after 48 hours incubation of IW 240, and the wild-type 23D5; although it is not possible with an obligate parasite, such as powdery mildew, to ensure identical levels of infection, these were not greatly different between the two strains. The wild-type produced more than three times as many conidia as IW 240. Similar observations were made with laboratory induced mutants.

Table 4. Conidial production by barley mildew and quinoxyfen sensitivity.

Strain	Quinoxyfen sensitivity ED_{80} mg/litre^{-1}	Conidia per leaf
23D5 (Wild-type)	0.06	$1.160 \pm 0.11 \times 10^6$
IW 240	>5.00	$0.358 \pm 0.12 \times 10^6$

The lower conidial production by IW 240, and related laboratory produced mutants, is likely to be one reason for the reduced pathogenicity of these 'resistant' strains. The normal development of conidiophores in the wild-type strain (23D5) was compared by scanning electron microscopy with the 'resistant' strain IW 240. Unlike 23D5, the 'resistant' strain IW 240 produced no conidiophores six days after inoculation, confirming the observation that it sporulates poorly.

CONCLUSIONS

No cross-resistance was found between quinoxyfen and a representative azole (triadimenol). The baseline sensitivity (ED_{50}) of 340 field isolates of *E. graminis* f.sp. *hordei* to the fungicide quinoxyfen ranged from <0.0001-0.016 mg/litre, with a median of 0.003 mg/litre. These isolates were all obtained during 1991-1994, when the compound was under development and not available commercially. This broad range in baseline

sensitivity was similar to that observed for other mildew fungicides (Hollomon, 1975; DeWaard, 1994). Barley mildew isolates with ED_{50}s of >0.16 mg/litre were isolated with a low frequency from treated crops, but could only be maintained on quinoxyfen-treated leaves. *E. graminis* f.sp. *hordei* strains with similar 'resistant' phenotypes could be generated in the laboratory after exposure to the chemical mutagen NTG. These mutants were also dependent on quinoxyfen for their continued maintenance in the culture collection. Detailed studies of both these mutants and the field 'resistant' isolates revealed that conidia production was reduced, largely as a result of poor conidiophore production. It was concluded that these isolates were of low fitness and consequently the potential of isolates, such as IW 240, to cause epidemics is limited.

Based on the available data a resistance management strategy has been drawn up:

(A) - Restriction of exposure of the pathogen by not developing seed treatments and not recommending foliar use after the first awns visible growth stage (BBCH49);
(B) - Developing pre-formulated mixtures and tank-mix recommendations with other fungicide groups of different modes of action;
(C) - alternation of quinoxyfen with fungicides of different modes of action when mildew control outside of the quinoxyfen window is needed.

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SELECTION FOR CARBENDAZIM RESISTANCE IN *FUSARIUM* SPECIES ON WHEAT AND RICE IN CHINA

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ABSTRACT

Fusarium graminearum and *Fusarium moniliforme* are important diseases of wheat and rice, respectively, in central and southern parts of China. Until recently, both diseases were well controlled with benzimidazole fungicides, but now they are only effective against wheat scab (*F. graminearum*). Carbendazim-resistant strains of both pathogens are easily generated in the laboratory, and occur in field populations, but their frequency has remained very low in *F. graminearum* populations despite intensive use of carbendazim for more than 20 years. In contrast, half the *F. moniliforme* isolates from rice crops are carbendazim-resistant. The mechanism of carbendazim resistance in *F. graminearum* is unknown, but it is controlled by a single gene, and seems likely to involve changes in β-tubulin. But unlike other pathogens, resistance does not involve changes at amino acid codons 198 or 200. Knowledge of the mechanism of resistance should help management of anti-resistance strategies for control of wheat scab.

INTRODUCTION

Some twenty different Fusarium species are pathogenic on wheat and rice in China. Especially important are *F. moniliforme* (= *Gibberella fujikuroi*) the cause of rice "bakanae" disease, and *F. graminearum* (= *Gibberella zeae*) which is the main component causing "wheat scab", or Fusarium ear blight. *F. graminearum* infects other cereals, and wind-blown ascospores produced on residual straw are the main source of inoculum each spring (Suty & Mauler-Machnik, 1996). Both these Fusarium species cause extensive yield losses and, in the case of *F. graminearum*, potent mycotoxins are produced in diseased grain. Consequently, wheat consignments with more than 4% of infected grain can no longer be used for animal or human consumption.

Both diseases were well controlled by the benzimidazole fungicide, carbendazim. Against wheat scab this requires two treatments, one at anthesis and the second a week later (Cook, 1981). For "bakanae" disease, seed treatment ensured that only healthy rice seedlings were transplanted. Intensive use of carbendazim over the past 20 years has been a major component in the management of Fusaria diseases in cereal growing regions, especially throughout China south of the Yangtze river valley.

In different crops world-wide, the outcome of selection with benzimidazole fungicides has been quite erratic, and although resistance has been a practical problem in several pathogen populations, Fusarium sp. have responded quite differently despite apparently similar levels of exposure. Benzimidazole resistance is widespread and significant in *F. nivale* (= *Microdochium nivale*) but not in *F. culmorum* (Locke *et al.*, 1987), despite the fact that both species are commonly isolated from scabbed grain (Parry *et al.*, 1995), and both pathogens will have been exposed to benzimidazoles, albeit applied at GS31 for eyespot (*Pseudocercosporella herpotrichoides*) control. In this paper the occurrence of resistance to carbendazim in both rice "bakanae" and wheat scab diseases is examined, and it is reported that the two Fusaria have responded differently to selection.

MATERIALS AND METHODS

Isolation, maintenance and assay of Fusarium isolates

F. graminearum and *F. moniliforme* were isolated from surface-sterilized diseased wheat ears or rice stems, respectively, by plating onto Potato Dextrose Agar (PDA) containing streptomycin and chloromycetin (both 100 units ml^{-1}) to prevent bacterial contamination. For routine monitoring, isolates were tested at this stage by transferring a disk (4 mm diam.) of actively growing mycelium onto fresh PDA at 25°C, containing a range of fungicide concentrations. Sensitivity was established from the dose-response relationship obtained by measurement of mycelial growth at each fungicide concentration. Isolates used in further studies were purified as single conidial isolates and stored on PDA at 4°C.

Mutation of *F. graminearum*

A conidial suspension of the wild-type sensitive strain JF18 (Table 3) was exposed 25 cm below a 20 Watt u/v light for up to 45 secs; this achieved 90-95% kill. Irradiated conidia were plated onto Potato Sucrose Agar containing carbendazim (10 μg ml^{-1}) and actively growing colonies transferred to unamended medium after 7-10 days at 25°C. Once the stability of any resistant mutants was established, they were maintained on PDA without added fungicide.

Crossing of *F. graminearum*

Ascospores were obtained from ascocarps produced from paired strains inoculated onto autoclaved rice straw. Unfortunately, *G. zeae* is self-compaztible and, as no suitable markers are available, ascospores from individual ascocarps were isolated and tested. Only ascocarps containing carbendazim-resistant and -sensitive ascospore progeny were assumed to be derived from crosses between two parental isolates. Progeny were maintained in the absence of fungicide, and tested at a single dose of carbendazim (10 μg ml^{-1}) to determine if they were resistant.

Recombinant DNA procedures

DNA was extracted from freeze-dried mycelium of both *F. graminearum* and *F.*

moniliforme grown in Czapek Dox liquid media for 5 d at 25°C. Dried mycelium was ground in a pestle and mortar with small glass beads (Sigma Company, Poole, Dorset, Cat. No. G1145) and extracted with phenol: chloroform as described by Wheeler *et al.* (1995). An 821 base pair fragment of the β-tubulin gene containing the coding sequence for amino acids 135-406 was amplified by Polymerase Chain Reaction (PCR) using two primers, B1 and B3, designed to consensus regions of fungal β-tubulin genes.

```
B1   5'  A  A  A  T  C  A  C  C  C  A  C  T  C  T  C  T  T  C  G  T  G  G  T  G  G  3'
            G        T                 T        C        C

B3   5'  C  T  C  C  A  T  C  T  C  G  T  C  C  A  T  G  C  C  C  T  G  A  C  C     3'
                                       A           T        G
                                       T
```

Amplification conditions involved denaturation at 94°C for 4 min, then 30 cycles of denaturation (94°C; 1 min) annealing (55°C; 1 min) and extension (72°C; 1 min). The amplified fragment was gel purified after Agarose (1.2% Nusieve) electrophoresis, and cloned into the Sma 1 site of the plasmid pUC18 using a "SureClone" kit according to the manufacturer's instructions (Pharmacia, St. Albans, UK). The inserts were sequenced using Sequenase (Amersham International, UK).

RESULTS

Sensitivity to carbendazim

One hundred isolates of both *F. graminearum* and *F. moniliforme* were obtained from regions of China where little or no fungicide has been used. The frequency distribution was unimodal, and although carbendazim sensitivity varied (Table 1), no isolates grew on PDA containing more than 4 μg ml^{-1} carbendazim.

Table 1. Base-line sensitivity of two Fusarium species to carbendazim

Species	No. of isolates	Mean ED_{50} of population μg ml^{-1}	Range of sensitivity μg ml^{-1}
F. graminearum	100	0.56 ± 0.09	$0.3 \rightarrow 0.83$
F. moniliforme	100	0.65 ± 0.10	$0.37 \rightarrow 0.98$

Following reports of poor control of "bakanae" disease with carbendazim, a monitoring programme was initiated, where fungicide use was intensive. A single discriminating dose of 4 μg ml^{-1} of carbendazim identified resistant isolates. Between 25% and 70% of *F. moniliforme* isolates were benzimidazole resistant (Table 2). More detailed analysis of some isolates identified three resistant phenotypes: (i) = MR (moderately resistant; Minimum Inhibitory Concentration (MIC) < 20 μg ml^{-1}); (ii) = HR (highly resistant; MIC = 21-100 μg ml^{-1}); (iii) VHR (very highly resistant; MIC > 100 μg ml^{-1}). Only 63 field isolates of *F. graminearum* were resistant, but not all were stable in the absence of fungicide and resembled wild-type when assayed after several generations on unamended PDA.

Table 2. Frequency of carbendazim-resistant isolates of Fusarium from field crops*.

	F. graminearum			F. moniliforme		
	No of isolates tested	Resistant	% Frequency	No of isolates tested	Resistant	% Frequency
1992	405	1	0.25	57	40	70
1993	1040	13	1.25	300	140	46
1994	940	17	1.81	-	-	-
1995	1900	5	0.79	981	245	25
1996	788	17	2.16	-	-	-

* Data for *F. graminearum* from Zhejiang province and for *F. monileforme* from Jiangsu, Zhejiang and Liaoning provinces.

Laboratory mutation and cross resistance

Carbendazim-resistant mutants of *F. graminearum* were obtained following u.v. irradiation of conidia with a frequency of 1×10^{-6} to 1×10^{-7}. Twenty mutants were characterised in more detail. All were classified as HR phenotypes, were stable in the absence of fungicide, grew and sporulated at the same rate of the parent, and were pathogenic on wheat. Zhou *et al.*(1994) have described these mutants in more detail.

Regardless of their source, all mutant isolates of *F. graminearum* showed cross resistance to benomyl, thiabendazole and thiophanate-methyl but increased sensitivity to methyl dichlorophenyl carbamate (MDPC). HR and VHR isolates of *F. moniliforme* showed this negative cross-resistance with MDPC, whereas MR isolates did not.

Table 3. Cross resistance patterns in Fusarium

	ED$_{50}$ μg μl^{-1}				
	carbendazim	benomyl	thiabendazole	thiophate-methyl	MDPC
F. graminearum					
JF18 Wild-type	0.3	0.9	0.8	5.4	30.0
JF 18R Mutant	3.9	5.3	4.8	52.6	4.2
2F 341 Field	21.2	NT	NT	NT	12.7
F. moniliforme					
N 76 Wild-type	0.5	NT	2.1	NT	27.5
M 80 MR	2.0	NT	2.1	NT	26.0
M 81 HR	22.5	NT	10.6	NT	4.2
M 82 VHR	57.5	NT	>100	NT	5.3

NT = Not tested

Genetics of carbendazim resistance in *F. graminearum*

Fifty-six perithecia were analysed from a mixed infection of a carbendazim-resistant and a sensitive strain. The majority contained only sensitive or resistant progeny, but 89 ascospore progeny from one perithecium segregated in their sensitivity to carbendazim as 1:1 (44:45). This indicated that the perithecium developed from a cross between the two parental strains, that resistance was a heritable character and controlled by a single gene. A similar analysis of perithecia from a mixed infection of two resistant strains yielded only resistant progeny, suggesting that if more than one resistance allele was present, it occurred at the same locus.

β-tubulin and carbendazim resistance in *F. graminearum*

Although several different amino acid changes in the β-tubulin confer carbendazim resistance in laboratory mutants of many fungi, in field strains of pathogens, resistance is correlated with alterations at amino acid codons 198 or 200 (Hollomon & Butters, 1995). Sequence analysis of the 821 base pair fragment of the β-tubulin gene of *F. graminearum* showed that the amino acid sequence in this region of β-tubulin of resistant strains was no different from that of a wild-type (Table 4).

Table 4. Amino acid sequence from codons 194 to 202 in *F. graminearum* strains

Strain	Amino acid codon								
	194				198		200		202
JF12 wild-type	Glu	Asn	Ser	Asp	**Glu**	Thr	**Phe**	Cys	Ile
JF12R laboratory-resistant mutant	Glu	Asn	Ser	Asp	**Glu**	Thr	**Phe**	Cys	Ile
FG2F269 field-resistant strain	Glu	Asn	Ser	Asp	**Glu**	Thr	**Phe**	Cys	Ile
DNA sequence	GAG	AAC	TCC	GAC	GAA	ACT	TTC	TGT	ATC

DISCUSSION

It is clear that benzimidazole-resistant strains of both *F. graminearum* and *F. moniliforme* can easily be generated in the laboratory, and occur in field populations of both pathogens in China. Yet only in rice "bakanae" disease has resistance become a practical problem leading to poor performance, even though both pathogens have been exposed to intensive use of carbendazim. In the Chinese regions monitored in this study, wheat and rice are grown in succession and populations of *F. graminearum*, which occur in both crops, are exposed to at least three treatments each year. Despite this, control of wheat scab with carbendazim was still very effective in 1995 (Zhou, unpublished result).

The mechanism of benzimidazole resistance in these two Fusaria has not been

established, but negative-cross resistance occurs between benzimidazole and $\underline{N}$-phenyl carbamate (MDPC) fungicides suggesting that changes in the target β-tubulin are involved. Unlike other plant pathogens, resistance in *F. graminearum* is not correlated with changes at amino acid codons 198 or 200, but presumably from changes elsewhere in the protein. Understanding the mechanism of this resistance in *F. graminearum,* and why the mechanism is not selected in field populations, should help long-term management of anti-resistance strategies for wheat scab control.

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A BASELINE FOR THE SENSITIVITY OF *BOTRYTIS CINEREA* TO PYRIMETHANIL

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ABSTRACT

A microtitre assay was used to determine the sensitivity to pyrimethanil of 623 isolates of *Botrytis cinerea*. The isolates were obtained during 1995 from a total of 73 locations in France, Switzerland and Spain where pyrimethanil had never been applied. French and Swiss isolates were from grapevines, while those from Spain were from tomato crops. The sensitivity distribution of the Spanish isolates was significantly different from those of the other two countries, which were not significantly different from each other. There were slight differences between distributions of isolates from five different wine-growing regions of France but no regional differences were seen in Swiss isolates.

INTRODUCTION

Pyrimethanil is a new anilinopyrimidine fungicide which has recently been introduced for the control of *Botrytis cinerea* in grapevines and other susceptible crops (Neumann *et al.*, 1992). Pyrimethanil has a novel mode of action, shared with other members of the anilinopyrimidine group, in which inhibition of extra-cellular enzyme secretion and amino acid biosynthesis have been found to be involved (Milling and Richardson, 1995, Miura *et al.*, 1994, Masner *et al.*, 1994, Leroux, 1994). Laboratory experiments have established that pyrimethanil is highly active against all strains of *B. cinerea*, including those which are resistant to other botryticides, such as dicarboximides, benzimidazoles and N-phenylcarbamates (Leroux & Montcomble, 1993). Resistance to these groups developed relatively rapidly after their introduction and *Botrytis* can, therefore, be regarded as a high-risk pathogen. AgrEvo undertook the development of a resistance management strategy as an essential step in the commercialization of pyrimethanil. This has been accomplished by the recommendation that the product should always be used in a programme with other botryticides with different modes of action and that the frequency of application should be limited, in line with guidelines formulated by the Fungicide Resistance Action Committee (FRAC).

In order to monitor the effectiveness of this strategy, it is essential to have a suitable method for testing the sensitivity of *Botrytis* populations and to know the sensitivity of populations which have never been treated with the fungicide, thereby establishing a baseline against which suspected changes in sensitivity can be measured (Brent, 1995).

In order to fulfil these requirements, a monitoring method was developed over several years (Birchmore et al., 1996, Birchmore and Williams, 1996). This was used to quantify the levels of sensitivity of isolates derived from French and Swiss grapevine crops and Spanish tomato crops which it was known had never been treated with anilinopyrimidines. In addition, the study was also intended to give information on whether sensitivity to pyrimethanil is evenly distributed geographically and in different crops.

MATERIALS AND METHODS

The method used to determine the sensitivity of Botrytis isolates to pyrimethanil was that described by Birchmore and Williams (1996).

Sample collection

Samples of Botrytis conidia were obtained from sporulating, infected grape berries by means of sterile cotton swabs. These were sealed individually into sterile plastic tubes and returned to the testing centre by the fastest possible means. Ten sample swabs were taken from each location.

Inoculum production

The conidia on each swab were sub-cultured in a vertical laminar-flow hood, by tapping on the edge of a 9.0 cm Petri dish containing the medium of Last & Hamley, (1956), which was used for isolation and induction of sporulation. The Petri dishes containing the isolates were incubated in the dark at 18°C for approximately four days, then illuminated with near u.v. light for a further five to six days at the same temperature. Conidia from the sporulating culture were suspended in sterile Glucose-Gelatin (G-G) medium which was filtered to remove fragments of mycelium and agar before adjustment to 10^5 conidia per ml.

The G-G medium used consisted of 1.75g of $KH_2 PO_4$, 0.75g of $Mg SO_4$, 4.0g of glucose and 4.0g of gelatin per litre of distilled water.

Test preparation and incubation.

Technical pyrimethanil was dissolved in dimethylsulphoxide (DMSO). This stock solution was used to prepare a range of concentrations of pyrimethanil in sterile G-G medium. Equal volumes (0.1ml) of spore suspension and pyrimethanil solution were mixed in the wells of sterile microtitre plates. The final concentrations of pyrimethanil were 50.0, 12.5, 5.0, 2.5, 1.25, 0.5, 0.25, 0.05 and 0 ppm. The concentration of DMSO in all of these final test solutions was kept constant at 2.0 %. The microtitre plates were incubated in the dark at 18°C.

Assessment method

The degree of germ tube growth which occurred in the test solutions was assessed by measuring their optical density at 0 and 48 h using a Dynatech plate reader at a wavelength of 590 nm.The optical density readings for each isolate and concentration of pyrimethanil were

converted to the fold increase over the 48 hours of incubation, by comparison with the 0 hour data. These fold increase values were then used to plot the dose response to pyrimethanil of each isolate and from this an IG_{50} (50% Inhibition of Growth) value was calculated. This corresponded to the concentration which was calculated to give 50% reduction in fungal germ tube growth over the 48 hours of incubation.

RESULTS AND DISCUSSION

The sensitivity to pyrimethanil of a total of 623 isolates of *B. cinerea* were evaluated from 73 sites in 3 countries. Those from France were from five major wine-producing areas, the Swiss ones from two vine regions, the area around Lake Geneva and between Basle and Zurich, while the Spanish tomato samples were from the Almeria region. The cumulative frequency distributions of these IG_{50} values for each of the three countries are shown in Figure 1.

Figure 1. Cumulative frequency distributions of IG_{50} values for sensitivity to pyrimethanil of *Botrytis cinerea* isolates from French and Swiss grapes and Spanish tomatoes.
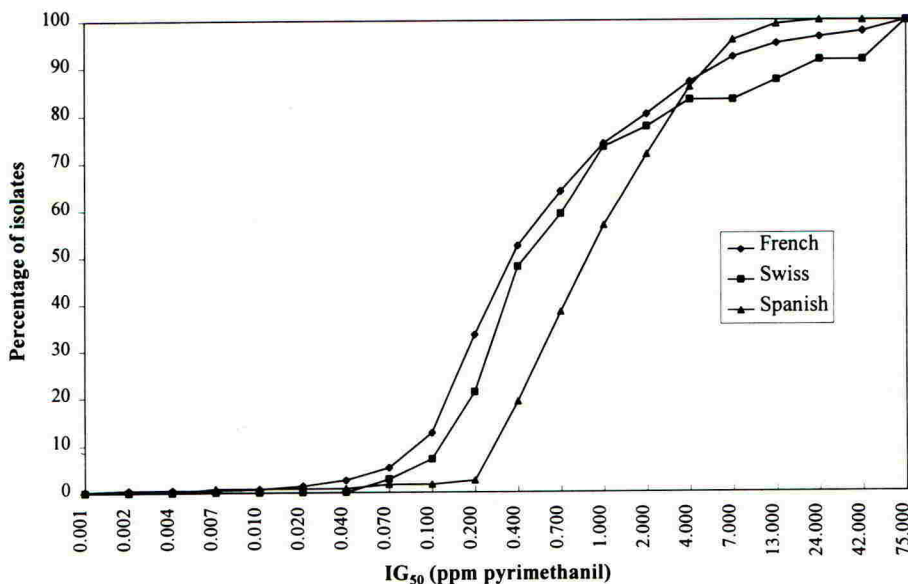
In each of the cases the distributions were relatively broad. The curves given by the isolates from French and Swiss grapes were very similar and in fact were found to be not significantly different, using the Kolmogorov - Smirnov non-parametric test (Gibbons, 1976) to compare distributions. However, the distribution of results obtained from Spanish tomato crops appeared to be different from the other two. This was confirmed when the IG_{50} values were ranked and compared using another non-parametric test, the Kruskal-Wallis one way analysis of variance (Gibbons, 1976). The results of this latter procedure are shown in Table1.

Table 1. Numbers of sites and *Botrytis* isolates from France, Switzerland and Spain tested during 1995 sensitivity monitoring. Results of statistical analysis of differences between distributions.

Country	Numbers of sites	Numbers of isolates tested	Median IG_{50} ppm	Mean rank $p = < 0.0001$
France	52	422	0.54	281.4
Switzerland	9	71	0.61	312.7
Spain	12	120	1.56	391.7
Totals	73	623		

This difference could be due to a number of factors affecting the sensitivity of *Botrytis* populations to fungicides. The greater geographical separation of the Spanish population from those in France and Switzerland could be responsible, perhaps reflected in the relative efficiency of the Alps and Pyrenees as barriers to the movement of *Botrytis* conidia. In addition, climatic factors could have an influence, although the most likely explanation for the difference is that the French and Swiss samples were drawn from vine crops, while those from Spain were taken from tomatoes. Further work is planned to investigate this finding.

The samples of French *Botrytis* conidia were taken from crops in five different wine-producing regions of the country, as shown in Table 2.

Table 2. Departments in the five French regions from which *Botrytis* samples were obtained during 1995.

Region	Bordeaux	Muscadet	Champagne	Val-de-Loire	Bourgogne/ Beaujolais
Departments	Gironde.	Loire-Atlantique.	Marne.	Maine-et-Loire. Vienne.	Côte-d'Or. Rhône. Saône et Loire.

The distributions of IG_{50}s of isolates from the five regions are shown in Figure 2. Although the curves were very similar, there was a significant difference between them, according to the results of Kruskal - Wallis non-parametric one-way analysis of variance (Gibbons, 1976) (Table 3).

Figure 2. Cumulative frequency distributions of IG_{50} values for sensitivity to pyrimethanil of *B. cinerea* isolates from five wine-producing regions of France.
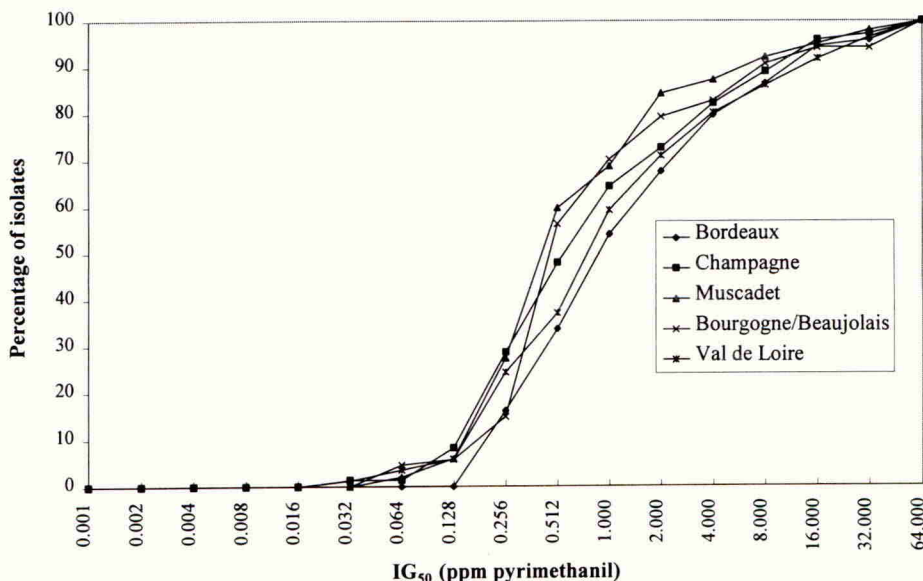

Table 3. Numbers of sites and *Botrytis* isolates from five regions of France, tested during 1995 sensitivity monitoring. Results of statistical analysis of differences between distributions

Region	Numbers of sites	Numbers of isolates tested	Median IG_{50} ppm	Mean rank p - value = 0.04
Bordeaux	11	74	0.83	243.3
Muscadet	10	102	0.38	187.8
Champagne	11	73	0.54	204.4
Val de Loire	10	86	0.63	223.6
Bourgogne/ Beaujolais	10	87	0.44	206.1

Pairwise comparisons, using an "experiment error rate" of 0.1 suggested that the significant difference was due to that between the isolates from Bordeaux and those from Muscadet, but this was so borderline that it could have been spurious. The differences were much less marked than that seen between the Spanish isolates and those from France and Switzerland, indicating that variations within a country were relatively minor, even between separate areas of production.

No such differences were seen when the Swiss isolates were grouped into those from the French-speaking region, near Lake Geneva and those from the more northern, German-speaking area, between Basle and Zurich.

In conclusion, the work carried out during 1995 established baseline distributions for the sensitivity of French, Swiss and Spanish *Botrytis* populations to pyrimethanil, against which the sensitivity of isolates from treated crops can be measured in subsequent years. The baseline will be extended to other countries and crops, in line with the broadening registration and commercialization of pyrimethanil for *Botrytis* control.

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STUDIES INVESTIGATING THE SENSITIVITY OF *PHYTOPHTHORA INFESTANS* TO PROPAMOCARB HYDROCHLORIDE

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ABSTRACT

Following the recent introduction of propamocarb based products for the control of potato late blight (*Phytophthora infestans*) a sensitivity monitoring programme was instigated in 1994 and continued in 1995. Results from 1994 showed that propamocarb is equally effective against phenylamide sensitive and insensitive strains, and that there was no difference in the response to propamocarb of A1 and A2 mating type isolates. Results from 1995 confirmed those from 1994 and in addition showed that there was no change from 1994 in the overall sensitivity of isolates to propamocarb.

INTRODUCTION

Propamocarb hydrochloride is a systemic fungicide introduced in 1980 for the control of oomycete pathogens in horticultural markets. Since 1993 propamocarb in combination with mancozeb 'Tattoo'® or chlorothalonil 'Tattoo C'® has been introduced for the control of potato late blight (*Phytophthora infestans*) in Europe and the Americas (Löchel & Birchmore, 1990).

Phytophthora infestans is a pathogen well known for its rapid development of resistance to various anti-metabolites. This was particularly the case with the phenylamide group of compounds which were temporarily withdrawn from use in several countries during the early 1980's following several treatment failures, and were only reintroduced after an anti-resistance strategy was put in place (Davidse *et al*, 1989). The effectiveness of this strategy has allowed this group of compounds to continue providing an effective contribution in the control of potato late blight.

As part of a pre-emptive anti-resistance strategy for propamocarb, a sensitivity monitoring programme was instigated in 1994 at the University of Wales, Bangor. The purpose of the programme in 1994 was to investigate the sensitivity of a cross section of isolates to propamocarb, including both phenylamide sensitive and insensitive strains. The sensitivity of the A1 and A2 mating types was also evaluated. In 1995, the programme concentrated on isolates from that year in

order to assess if there had been a change in the overall sensitivity of isolates to propamocarb.

MATERIALS AND METHODS

Isolates used were collected from sites in England, Wales, Northern Ireland, Republic of Ireland and Germany, and were cultured initially on rye A agar supplemented with rifamycin, ampicillin and nystatin which ensured that all fungal and bacterial contamination was removed. Isolates collected in years previous to 1994 were routinely stored in liquid nitrogen, whereas isolates received during 1994 and 1995 were maintained on rye A agar.

A leaf disk assay based on the FRAC methodology (Sozzi *et al.*, 1992) was used to determine the sensitivity of the isolates. Replicates of 5 leaf disks (13 mm diameter) of the late blight sensitive potato cultivar Home Guard were floated on solutions of propamocarb at concentrations of 0, 10, 50, 200 and 500 ppm a.i., each in a separate well of a square 25 well repli-dish. Prior to infection of the leaf disks, each isolate was passaged through Home Guard leaves to ensure that the isolates had retained pathogenicity. These leaves were used to prepare a suspension of $2\text{-}3\times10^4$ sporangia/ml, which was then incubated for 2-3h at 10° C to induce release of zoospores. Leaf disks were then inoculated with 20µl of the suspensions and incubated at 18° C for 7d using a photoperiod of 16 h per day.

Sporulation in 1994 and 1995 programmes was scored using the following scale:

Score	Observation
0	sporangiophores absent
1	1-4 sporangiophores per disk
2	5-12 sporangiophores per disk
3	moderate sporulation; sporangiophores visible under binocular microscope
4	profuse sporulation visible with the naked eye

The viability of sporangia from propamocarb treated potato plants was evaluated using excised Home Guard leaflets dipped in a 500 ppm propamocarb solution. After 2h the leaflets were inoculated with 20µl of a sporangial suspension as used in the leaf disk assay, 5 leaflets were used per treatment and assessed after 7d on the basis of leaf infection and sporulation. Any spores collected were then used to inoculate a further set of untreated potato leaves.

More than 100 isolates were examined in each year. Isolates tested in 1994 were predominantly from England and Wales and had been collected during the years 1978-1994. They had not been previously exposed to propamocarb. Isolates tested in 1995 came from England, Wales, Northern Ireland, Republic of Ireland and Germany and were collected in the years 1993 (6 isolates), 1994 (17 isolates)

and 1995 (84 isolates). It was not known if these isolates had previously been exposed to propamocarb.

Statistical analysis of the data was carried out using the Mann-Whitney non-parametric test (Mann & Whitney, 1948). This test was selected as the most suitable test of significance, as it is able to compare groups of differing sizes. In addition, the sensitivity ratio between the phenylamide sensitive and insensitive groups and the A1 and A2 mating types was calculated for each concentration of propamocarb.

RESULTS AND DISCUSSION

1994 monitoring

Data for the sensitivity distribution of isolates for the 1994 monitoring are shown in Table 1.

Table 1. Summary of median scores grouped by phenylamide sensitivity and mating type for sensitivity to propamocarb

	propamocarb ppm						
	0	10	50	100	200	500	No of Isolates
Phenylamide sensitive	4.0	4.0	3.0	1.9	1.0	0.4	82
Phenylamide insensitive	4.0	4.0	3.2	2.3	1.2	0.4	38
Mating type A1	4.0	4.0	3.0	2.0	1.0	0.2	103
Mating type A2	4.0	4.0	3.1	2.5	1.3	0.7	18
Overall	4.0	4.0	3.0	2.0	1.0	0.4	123

Owing to the non-quantifiable scoring system, median values rather than mean values were used to summarise the raw data. It should be noted that phenylamide sensitive and insensitive isolates can be either A1 or A2 mating types.

In Table 2 data are presented comparing the propamocarb sensitivity of phenylamide sensitive and insensitive strains.

Table 2. Summary of the statistical analysis comparing phenylamide sensitive and insensitive groups of isolates of *Phytophthora infestans*

| ppm propamocarb | Sensitivity Ratio | p value | Mann-Whitney Test | |
			Median Difference	95 % Confidence Interval
10	1.00	0.352	0.0	0 to 0
50	1.07	0.193	0.2	-0.2 to 0.6
100	1.21	0.148	0.2	-0.2 to 0.8
200	1.20	0.472	0.0	-0.4 to 0.4
500	1.00	0.453	0.0	0 to 0.2

At all the concentrations used there was no significant difference in the sensitivity of the two groups to propamocarb. Thus propamocarb based products should be equally effective against phenylamide sensitive and insensitive strains in field situations.

A breakdown of the analysis of propamocarb sensitivity according to mating type is shown in Table 3.

Table 3. Summary of the statistical analysis comparing A1 and A2 mating types of *Phytophthora infestans*

| ppm propamocarb | Sensitivity Ratio | p value | Mann-Whitney Test | |
			Median Difference	95 % Confidence Interval
10	1.00	0.192	0	0 to 0
50	1.03	0.367	0	-0.4 to 0.6
100	1.25	0.443	0	-0.6 to 0.8
200	1.30	0.318	0	0.4 to 0.8
500	3.50	0.311	0	0 to 0.6

As with the previous groups (Table 2) there was no significant difference between A1 and A2 mating types in their response to propamocarb

Two isolates with scores above and below the median values were selected for further testing to evaluate the viability of sporangia after propamocarb treatment, and the results are shown in Table 4.

Table 4. Sporulation (sporangia per lesion) of 2 isolates of *Phytophthora infestans* inoculated onto Home Guard leaves treated with 500 ppm propamocarb and subsequently transferred to untreated leaves

| | Treated leaves | | Untreated Leaves | |
	control	propamocarb	control	propamocarb
Isolate 1	0.4 ± 0.18	0.0	-	-
Isolate 2	12 ± 0.71	0.1 ± 0.05	1.0	0.0

Isolate 1 had previously shown very high sensitivity to propamocarb in the leaf disk assay, whereas Isolate 2 had been less sensitive. From the results in Table 4 it appears that Isolate 2 is more aggressive than Isolate 1, but that it would be effectively controlled by propamocarb in the field as the spore production is reduced to a non-viable level.

1995 monitoring

In 1995, no comparison could be made between the sensitivity of A1 and A2 mating types, as only one A2 isolate was collected throughout the year. The scarcity of A2 isolates was a common observation across the whole of Europe during 1995 (U Gisi, personal communication).

The 1995 monitoring data comparing the phenylamide sensitive and insensitive isolates are summarised in Table 5.

Table 5. Summary of median scores grouped by phenylamide sensitivity of isolates of *Phytophthora infestans*

| | propamocarb ppm | | | | | | |
	0	10	50	100	200	500	No of Isolates
Phenylamide sensitive	4.0	4.0	2.0	1.2	1.0	0.2	45
Phenylamide insensitive	4.0	4.0	3.0	2.2	1.2	0.2	65
All isolates	4.0	4.0	3.0	2.0	1.0	0.2	113

Statistical analysis of the 1995 data comparing phenylamide sensitive and insensitive isolates is summarised in Table 6.

Table 6. Summary of the statistical analysis comparing phenylamide sensitive and
insensitive groups of isolates of *Phytophthora infestans*

		Mann-Whitney Test		
ppm propamocarb	Sensitivity Ratio	p value	median difference	95 % Confidence Interval
10	1.00	0.015	0.0	-0.2 to 0.0
50	1.50	0.033	-0.6	-1.0 to 0.0
100	1.83	0.199	-0.2	-1.0 to 0.0
200	1.20	0.489	0.0	-0.6 to 0.4
500	1.00	0.533	0.0	-0.2 to 0.0

At 10 and 50 ppm the results were significantly different at $p = 0.05$ but were not
significantly different at $p = 0.01$. At all other concentrations there was no significant
difference between the phenylamide sensitive and insensitive groups. Concentrations of
10 and 50 ppm propamocarb are not expected to have high activity against either
phenylamide sensitive or insensitive isolates, and it is probable that differences in the
pathogenicity of isolates are the cause of the differences seen at these concentrations.

Monitoring of propamocarb sensitivity in *Phytophthora infestans* has continued in 1996
and will be expanded in 1997 to cover the Americas and a wider spread in Europe.

ACKNOWLEDGMENTS

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EVOLUTION OF GRAPE POWDERY MILDEW INSENSITIVITY TO DMI FUNGICIDES

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ABSTRACT

Resistance of grape powdery mildew to fungicides that inhibit the C-14 demethylation of sterols (DMI) is an important problem in European vineyards. Field trials indicate that populations show a quantitative evolution during the season after treatments with DMIs. Through the years, two kinds of evolution are observed according to the principal form of overwintering for the fungus: mycelium in buds or cleistothecia. When *Uncinula necator* survives winter as mycelium in dormant infected buds, populations collected in the beginning of the season from flag shoots are more sensitive than those sampled the previous year at the end of the season. When cleistothecia are present, populations maintain their level of resistance characterised the previous year.

INTRODUCTION

The first case of resistance of *Uncinula necator* to DMI fungicides was detected in 1988 (Steva *et al*, 1988). Today, in all European countries, the presence of resistant populations has been demonstrated. These populations could break down the efficacy of DMIs. To cope with this resistance, it is essential to develop strategies that will limit the emergence and multiplication of resistant phenotypes.

Across the season, the evolution of the grape powdery mildew resistance to DMIs is a quantitative phenomenon, characterised by a progressive shift of the sensitivity of the populations, followed by the appearance of new phenotypes more resistant (Steva, 1992). Through the years, the evolution of resistance might be affected by the epidemiology of grape powdery mildew. Indeed, two modes of overwintering occur in European vineyards: mycelium in dormant buds or cleistothecia. Cleistothecia can form very early in the season after the fusion of two compatible hyphae (Gadoury & Pearson, 1988). In order to determine the respective influence of the two modes of overwintering on resistance, evolution of population sensitivities has been monitored on several experimental field plots since 1989. Populations have been sampled at different periods. The method used to characterise the population sensitivity to DMIs has been described previously (Steva, 1994). It is based on the culture of *U. necator* on leaf discs either treated or untreated, and observation of the individual hyphal growth of the germ tube produced by conidia 3 days after inoculation.

OVERWINTERING OF THE FUNGUS AS MYCELIUM IN BUDS

Figure 1 shows the evolution of *U. necator* population sensitivity in 1991 and 1992 in two vineyards where the fungus only survives winter as mycelium in dormant infected buds. In Narbonne, a shift in the sensitivity of the populations during the seasons can be seen. At the end of the year, the populations are resistant and the minimum inhibitory concentration (MIC) values are close to 10 mg/litre in 1991 and 30 mg/litre in 1992. Nevertheless, at the first sampling dates (14 May 1991 and 12 May 1992), the populations have the same level of sensitivity: MIC values is between 1 and 3 mg/litre of triadimenol. In Perpignan, the population

is sensitive in May and, progressively the percentage of less sensitive spores increases and resistant phenotypes emerge.
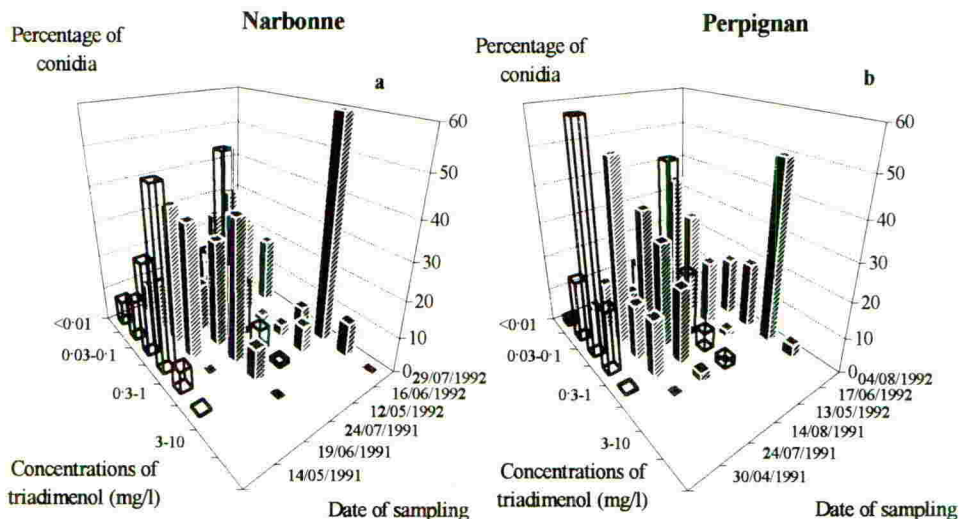

Figure 1. Evolution of *Uncinula necator* population sensitivity to triadimenol during two years (1991 and 1992) after treatment with triadimenol alone (37·5 g a.i./ha) in two vineyards with flag shoots : **a**. Narbonne **b**. Perpignan

In Table 1, LC_{50} values calculated for each sampling have been reported for the site of Narbonne. They confirm the progressive shift during the years. First, the LC_{50} value is close to 0·1 mg/litre in 1991 and 1992 but in July, LC_{50} value is 0·5 mg/litre in 1991 and 2 mg/litre in 1992. In Perpignan (Table 2), LC_{50} gets to 0·3 mg/litre of triadimenol. Next year, LC_{50} is 0·15 mg/litre. At the end of the treatments, the decrease of sensitivity is marked by a LC_{50} value near to 1·4 mg/litre.

Table 1. LC_{50} values (expressed in mg/litre of triadimenol) of *Uncinula necator* populations collected during two years in an experimental plot treated with triadimenol located in Narbonne*

Locations	LC_{50} values (mg/litre of triadimenol)					
	14/05/1991	19/06/1991	24/07/1991	12/05/1992	16/06/1992	29/07/1992
Narbonne	0·164	0·105	0·527	0·123	0·211	1·917

*Vineyard with flag shoots in the begining of the season

Table 2. LC_{50} values (expressed in mg/litre of triadimenol) of *Uncinula necator* populations collected during two years in an experimental plot treated with triadimenol located in Perpignan*

Locations	LC_{50} values (mg/litre of triadimenol)					
	30/04/1991	24/07/1991	14/08/1991	13/05/1992	17/06/1992	04/08/1992
Perpignan	0·079	0·173	0·267	0·147	0·078	1·384

*Vineyard with flag shoots in the begining of the season

OVERWINTERING OF THE FUNGUS AS CLEISTOTHECIA

Two vineyards without flag shoots were studied between 1990 and 1993: Azambuja (Portugal) and Perpignan (France). The plots were treated with triadimenol (registered rates). In Azambuja (Figure 2.a), a constant evolution of the sensitivity of the population through the

years was observed. The frequency of the resistant phenotypes increased instead of the more sensitive ones. In Perpignan, the population has a MIC close to 10 mg/litre in the first sampling. At the end of this year, the presence of a new phenotype was detected.
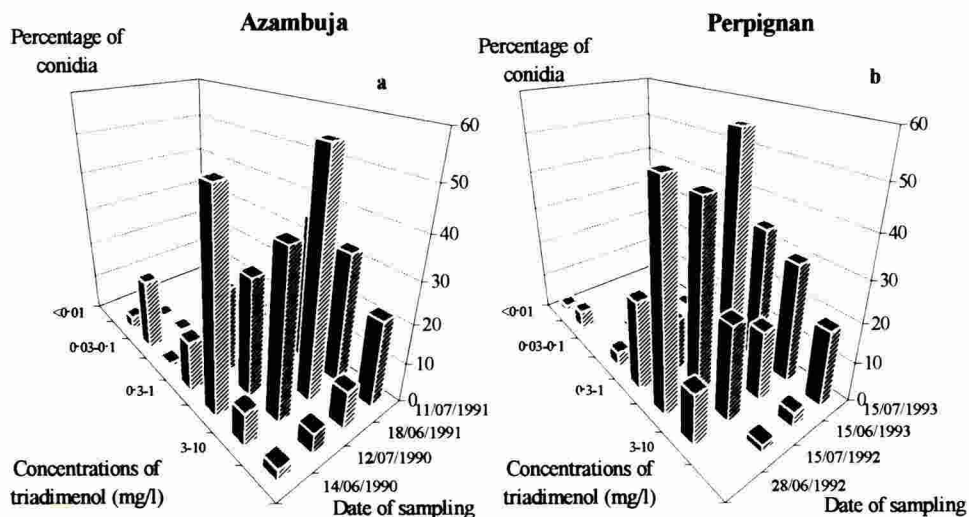

Figure 2. Evolution of *Uncinula necator* population sensitivity to triadimenol after treatment with triadimenol alone (50 or 37.5 g a.i./ha) in two vineyards with cleistothecia : **a**. Azambuja and **b**. Perpignan

LC_{50} values showed a progressive evolution in Azambuja (Table 3). At the beginning, the LC_{50} was close to 2.3 mg/litre and increased to 7.2 mg/litre by July 1991. The population was more resistant (LC_{50} = 5.81 mg/litre) in June 1991 than at the end of the previous year (LC_{50} = 4.2 mg/litre). In Perpignan (Table 4), comparable results were obtained.

Table 3. LC_{50} values (expressed in mg/litre of triadimenol) of *Uncinula necator* populations collected during two years in an experimental plot treated with triadimenol located in Azambuja*

Location	LC_{50} values (mg/litre of triadimenol)			
	14/06/1990	12/07/1990	18/06/1991	11/07/1991
Azambuja	2.282	4.247	5.81	7.133

*Vineyard with cleistothecia and no flag shoots in the begining of the season

Table 4. LC_{50} values (expressed in mg/litre of triadimenol) of *Uncinula necator* populations collected during two years in an experimental plot treated with triadimenol located in Perpignan*

Locations	LC_{50} values (mg/litre of triadimenol)			
	28/06/1992	15/07/1992	15/06/1993	15/07/1993
Perpignan	2.044	2.885	3.136	6.414

*Vineyard with cleistothecia and no flag shoots in the begining of the season

VINEYARDS WITH THE TWO MODES OF OVERWINTERING

Both flag shoots and cleistothecia were present in the two experimental vineyards: Rivesaltes and Trouillas. No DMI was applied on these plots during two years. But several DMIs had been sprayed in these locations and resistant populations were identified at the end of 1991.

In Rivesaltes (Figure 3), sensitive populations were found at the beginning of each season. This sensitivity, characterized by a MIC value under 1 mg/litre, was maintained up to the flowering stage (mid-June). The next sampling showed the presence of spores which were not controlled by doses less than 1 mg/litre. Some were only inhibited by 10 mg/litre. At the end of 1993, a very resistant phenotype appeared.
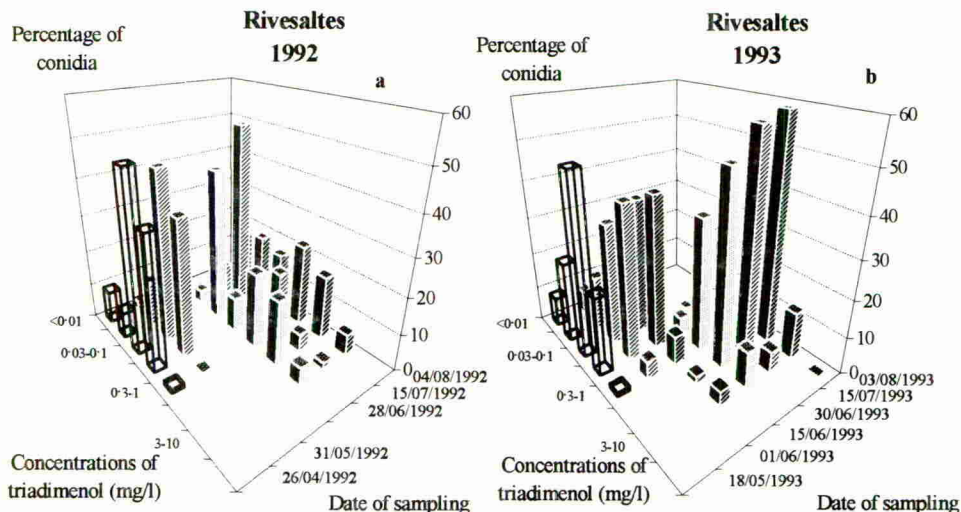

Figure 3. Evolution of *Uncinula necator* population sensitivity to triadimenol during two years (1992 and 1993) in a vineyard located in Rivesaltes (France) and untreated with fungicides
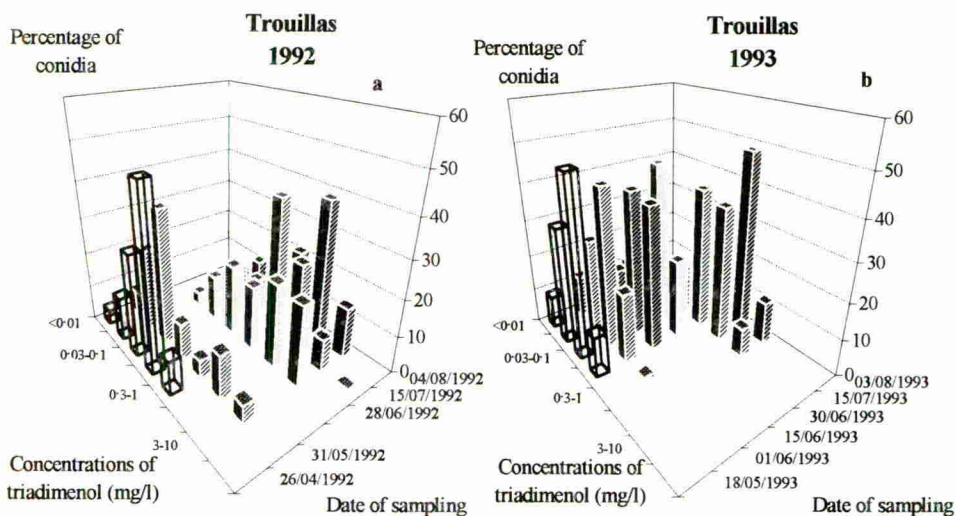

Figure 4. Evolution of *Uncinula necator* population sensitivity to triadimenol during two years (1992 and 1993) in a vineyard located in Trouillas (France) and untreated with fungicides

In Table 5, the LC_{50} is reported for 1992. In the beginning, values were close to $0\cdot1$ mg/litre. In Trouillas, the decrease in sensitivity was very fast. At the end, 50% of spores were only inhibited at $1\cdot8$ mg/litre. In Rivesaltes, the same efficacy was obtained with $0\cdot9$ mg/litre.

Table 5. LC_{50} values (expressed in mg/litre of triadimenol) of *Uncinula necator* populations collected in 1992 in two untreated vineyards : Rivesaltes and Trouillas (France)

Location	LC_{50} values (mg/litre of triadimenol)					
	26/04/1992	31/05/1992		28/06/1992	15/07/1992	04/08/1992
Rivesaltes	$0\cdot106$	$0\cdot109$		$0\cdot794$	$0\cdot393$	$0\cdot909$
Trouillas	$0\cdot166$	$0\cdot613$		$1\cdot952$	$1\cdot460$	$1\cdot790$

*Vineyards with flag shoots in the begining of the season and cleistothecia

Table 6. LC_{50} values (expressed in mg/litre of triadimenol) of *Uncinula necator* populations collected in 1992 in two untreated vineyards : Rivesaltes and Trouillas (France)

Location	LC_{50} values (mg/litre of triadimenol)					
	18/05/1993	01/06/1993	15/06/1993	30/06/1993	15/07/1993	03/08/1993
Rivesaltes	$0\cdot083$	$0\cdot132$	$0\cdot458$	$1\cdot791$	$1\cdot701$	$2\cdot126$
Trouillas	$0\cdot057$	$0\cdot073$	$0\cdot106$	$0\cdot076$	$0\cdot469$	$0\cdot591$

*Vineyards with flag shoots in the begining of the season and cleistothecia

A comparable phenomenon was observed in 1993 (Table 6). First, populations were sensitive ($LC_{50} < 1$ mg/litre). During the year, the evolution of resistance was progressive in Rivesaltes and, at the end, the LC_{50} was $2\cdot1$ mg/litre. In Trouillas, the shift was slower than in Perpignan.

CONCLUSIONS

These results allow the importance of the mode of overwintering of this fungus on the evolution of resistance to DMIs to be determined.

When the fungus only survives winter as mycelium in the buds, the populations are sensitive at the beginning of the season. If DMIs are not sprayed, the fungus keeps the same sensitivity throughout the year (Steva, 1994). On the other hand, an evolution in resistance is perceptible on treated plots. This decrease of sensitivity is not found in the beginning of the next year. This point tends to demonstrate that the maintenance of resistance in a vineyard is not linked to the presence of mycelium in buds. Young buds are infected with mycelium early in the season (Sall & Wrysinski, 1982, Van Der Spuy & Matthee, 1977) and the DMIs can't exert a selective pressure on this mycelium.

Samples collected on plots where cleistothecia represent the only mode of overwintering for grape powdery mildew, indicate that the selection exerted the previous year by the application of DMIs is perceptible at the beginning of the season from the emergence of the first symptoms.

The natural evolution of population sensitivities in the vineyards with the two modes of overwintering confirms these conclusions. In the beginning of the season, sampling realized on the first symptoms show the exclusive presence of sensitive populations whatever is the sensitivity of the population at the end of the preceding year. During the season, a brutal decrease of population sensitivity appears after the flowering stage. Originally, these vineyards have been treated with DMI fungicides for many years and a decrease of efficacy has been

observed. Thus, resistance is due to the exclusive use of DMIs in the preceding years. Dispersal of resistant conidia from treated plots to these untreated vineyards might be an explanation for this original shift. The introduction of exogenous resistant inoculum can be rejected as the source of resistance because movement of *U. necator* conidia between the plots is limited (Willocquet, 1994). The other hypothesis concerns the modes of overwintering of the fungus. This particular phenomenon can be explained by the release of ascospores induced by rain (Gadoury & Pearson, 1990).

Consequently, the importance of a good knowledge of the mode of overwintering of the fungus to promote anti-resistance strategies is recognized. In the absence of cleistothecia, DMIs can be sprayed several times in the season with a low risk of resistance the next year. The main recommendation is not to apply DMIs in the early stage of shoots growth, when the mycelium invades the young buds. In this case, there is no selection of resistant spores which can infect buds and give the first contamination the next year. Unfortunately, vineyards where cleistothecia are absent, are rare in Europe.

In all other cases, it is essential not to select excessively resistant spores during the season. The first cleistothecia can form early in the season on berries or on leaves. Most of the treatments, therefore, will have an influence on the sensitivity of inoculum that will be able to survive and emerge the next year. The best strategy consists in a limitation of DMIs to 2 or 3 treatments. One of the main questions that still needs to be answered concerns the period of spraying DMIs during the season. Specific experiments must be conducted as soon as possible to evaluate the relation between the number of DMIs, their period of spraying and the selective pressure against grape powdery mildew populations.

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BIOASSAYS FOR IMIDACLOPRID FOR RESISTANCE MONITORING AGAINST THE WHITEFLY *BEMISIA TABACI*

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ABSTRACT

The sweetpotato or cotton whitefly, *Bemisia tabaci*, is nowadays one of the most important homopteran pests on a variety of field and greenhouse crops. Imidacloprid, the first chloronicotinyl insecticide launched world-wide, is meanwhile registered in over 60 countries and shows excellent efficacy against a wide range of pest insects. It controls homopteran pests such as *B. tabaci* both by contact and, because of its systemic activity, by feeding. The efficacy of imidacloprid was investigated in adult leaf-dip bioassays, egg/larval-dip bioassays, systemic bioassays and a feeding bioassay on artificial double membranes (sachet-test). Whitefly populations from Almeria, Spain, showed a slight tolerance (2 to 5-fold) to imidacloprid compared to a reference laboratory strain in all bioassays used. A representative of the recently described *Bemisia* B-strains from cotton in California showed the same susceptibility to imidacloprid as the reference strain. Other insecticides tested were buprofezin, endosulfan, monocrotophos and pymetrozin, however, the highest resistance factors were determined for endosulfan and monocrotophos. Field trials in Almeria which were carried out between 1988 and 1996 have revealed no decline in the field performance of imidacloprid against *B. tabaci* in greenhouse vegetables to date.

INTRODUCTION

The sweetpotato or cotton whitefly, *Bemisia tabaci*, is an important homopteran pest of a vast range of field crops and greenhouse crops. It causes damage to crops by direct feeding, through the excretion of honeydew and furthermore as a vector of numerous viral pathogens. Agricultural losses exceeding half a billion dollars in the United States from 1991 to 1993 were attributed to *B. tabaci*, and a subtype of the genus *Bemisia* with a wider host range, the B-strain, also named *Bemisia argentifolii* (Perring *et al.*, 1993).

Resistance of *B. tabaci* to conventional insecticides such as organochlorines, organophosphates, carbamates and pyrethroids is now widespread and even to the very recently launched compounds pyriproxyfen and buprofezin resistance has been build up rapidly in certain areas of the world (Cahill *et al.*, 1995, 1996a; Horowitz & Ishaaya, 1994). The biochemical mechanisms conferring resistance to insecticides which have been described in detail included insensitive acetylcholinesterase, esterases and a point mutation in the GABA-gated chloride channel, the target of endosulfan (Byrne & Devonshire, 1993; Anthony *et al.*, 1995).

Among the compounds which entered the market very recently, the systemic chloronicotinyl insecticide imidacloprid is a very promising molecular tool in resistance management strategies. It controls resistant whiteflies and a range of other agricultural pests resistant to conventional

insecticide chemistry due to its excellent contact and oral activity (Elbert *et al.*, 1996). Imidacloprid acts agonistically on insect nicotinic acetylcholine receptors and shows only poor binding to vertebrate receptors (Methfessel, 1992).

Once a new compound like imidacloprid has been launched, its efficacy in the field on the different invertebrate target pests should be monitored carefully to recognize the possible build-up of resistance soon. Due to the high capability of *B. tabaci* to develop various mechanisms of resistance, a management strategy for any new whitefly material is essential. Therefore, the effects of imidacloprid and some other insecticides in different types of bioassays (contact and feeding) to elucidate the possible differences in mode of entry and to see which of the methods produced the most consistent and reliable data were studied. Some of the methods have already been described in detail, and were partially developed by M. Cahill at IACR Rothamsted. The bioassays have been validated on a susceptible laboratory strain. The resulting efficacy was compared to results retrieved when using field (greenhouse) populations from Almeria, Spain, which were regularly sprayed with imidacloprid in the past five years and one B-strain of *B. tabaci* from the United States.

MATERIALS AND METHODS

Whitefly strains

All strains of *Bemisia tabaci* were kindly provided by M Cahill (IACR–Rothamsted, Harpenden, UK). SUD-S was a susceptible laboratory strain. Two strains, ALM-2 and LMPA-2, were derived from greenhouse vegetables in Almeria, southern Spain, and reared in the laboratory since 1994 and 1995, respectively. CAL-1 was a typical B-strain from California, USA, collected from cotton in 1995. All strains were maintained on cotton plants (Cocker 312) at $25 \pm 1°C$, 60% r.h. and a photoperiod of L:D 16h:8h.

Insecticides

Imidacloprid, monocrotophos, and endosulfan were technical grade with a purity of higher than 95%. All other insecticides were used as commercial formulations: Buprofezin (Applaud 25WP), Pyriproxifen (Tiger 10EC) and Pymetrozin (Chess 25WP). Stock solutions of technical insecticides were prepared in acetone, whilst formulations were directly suspended in water. Serial dilutions of the insecticides were prepared in 0.02% (w/v) aqueous triton X-100 when using leaf-dip bioassays, in pure water when using the systemic test and in 15% (w/w) aqueous sucrose when using the sachet-test.

Adult leaf-dip bioassay

The procedure was used with slight modifications according to Elbert *et al.* (1996). Leaf discs (30 mm in diameter) were cut from cotton leaves and dipped in different concentrations of insecticide for 5 s. The leaf discs were then dried on filter paper in a ventilated hood for 20 min. The bases of small ventilated petri dishes (38 mm in diameter) were filled with 5 ml 1.2% agar gel. The leaf discs were placed on the agar with their upper side down. Adult whiteflies were removed from a rearing cage, anaesthetized briefly with CO_2 and were than spread onto a black-cloth covered ice-brick. Using a fine brush 20 adults (mixed sex) were transferred onto the treated leaf discs and the set-up was sealed with a ventilated lid. Directly after recovering from narcosis, adults were assessed for initial mortality and the dishes were

placed up-side down to simulate the normal feeding orientation of the whiteflies. Each bioassay was replicated at least three times using five to seven concentrations. Mortality was determined after 48 h.

Systemic bioassay

The procedure has already been published as one of the most suitable methods to assess the efficacy of imidacloprid against whiteflies (Cahill *et al.*, 1996b). Medium sized leaves were cut from cotton plants and the petioles were immersed in different concentrations of aqueous imidacloprid-solutions overnight (16 h under light in a ventilated hood at 21°C). Afterwards leaf discs (30 mm in diameter) were cut from the leaves and placed onto agar as described for the leaf-dip bioassay. Leaves immersed with their petioles in water alone were used for controls. The test was performed at least five times using two replicates each with five to six different concentrations. Percentage mortality was scored after 48 h.

Egg/larval-dip bioassay

One leaf of cotton plants with two true leaves was removed and the remaining one was cut to a size of c. 15 cm^2. These plants were infested with adult whiteflies (c. 150 per leaf) for 16 h. Afterwards the whiteflies were removed and the infested leaves were dipped in serial dilutions of imidacloprid for 5 s. Each set of plants were dipped at different time intervals to cover the different developmental stages, i.e. eggs, 1st instar, 2nd/3rd instar and the last instar. Percentage mortality was scored on representative leaf areas after the last larval instar has been reached (after 14-17 d, starting from the egg stage).

Sachet-test

The procedure of testing imidacloprid and other compounds with reasonable water solubility using artificial double membranes in a feeding bioassay has already been published for the resistance determination in aphids, when using insecticides which produce no plausible results in dip bioassays (Nauen *et al.*, 1996). This method was adopted for testing the susceptibility of whiteflies against imidacloprid and other insecticides after oral uptake. Each sachet (28 mm in diameter) consisted of two layers of stretched Parafilm which incorporated 0.4 ml of a 15% sucrose solution and different concentrations of insecticide. Adult whiteflies were handled as described above (leaf-dip bioassay). For each concentration of insecticide, 20 individuals were placed in appropriate containers and sealed with the corresponding double membrane. Directly after recovering from narcosis, the total number of living insects per unit was scored. Afterwards a piece of yellow cellophane was placed over the double membrane to enhance the feeding activity. Each concentration was tested in triplicate within a single bioassay consisting of at least five concentrations. Percentage mortality was assessed after 48 h at 21°C and 50% r.h..

LC$_{50}$-values and their 95% fiducial limits for all bioassays were calculated from probit regressions using the computer program POLO-PC (LeOra Software, Berkely, USA).

Field trials in southern Spain

Imidacloprid has been registered in Spain since 1992, developmental and registration trials had been conducted in the preceeding years. Trials were conducted between 1988 and 1996 in the regions of Almeria and Murcia. Applications of the commercial formulation Confidor 200 SL

at 0.01% a.i. were made in 750 - 1500l water/ha depending on the size of plants. A knap sack sprayer was used for the application. The plot size varied between 8 and 50 m² and each test was repeated four times. The infestation pressure was high at the first application and remained high throughout the trials. Evaluations of the experiments were carried out 8 to 17 days after the second application. The number of living larvae and nymphs on 40 leaves collected at random were assessed and the mortality - corrected by Abbott's formula - was calculated.

RESULTS AND DISCUSSION

Adult leaf-dip bioassay

When assessed in the leaf-dip bioassay, strains SUD-S and CAL-1 appeared slightly more susceptible to imidacloprid than strain ALM-2, which showed a resistance factor of c. 5 (Table 1). Strain ALM-2 has a marked resistance to endosulfan (Table 2). A resistance factor of 12 has been calculated for pymetrozin in strain ALM-2, but the leaf-dip test seems to be inappropriate for pymetrozin, because of a high variation of LC_{50}-values in the different replicates and low slope-values.

Table 1. Efficacy of imidacloprid against *B. tabaci* in adult leaf-dip bioassays

Strain	Imidacloprid $LC_{50(48h)}$ (ppm)	*95% FL	Slope	**RF
SUD-S	6.6	(4.5-9.3)	1.08	1
ALM-2	35	(24-51)	0.90	5
LMPA-2	15	(5.7-35)	1.16	2
CAL-1	8.6	(6.5-11)	1.13	1

*95% fiducial limits; **resistance factor = LC_{50} other strains / LC_{50} strain SUD-S

Table 2. Efficacy of endosulfan and pymetrozin against *B. tabaci* in adult leaf-dip bioassays

Insecticide	SUD-S $LC_{50(48h)}$ (ppm)	*95% FL	Slope	ALM-2 $LC_{50(48h)}$ (ppm)	*95% FL	Slope	**RF
Endosulfan	8.0	(6.4-10)	3.11	179	(152-210)	3.16	22
Pymetrozin	6.6	(3.6-12)	1.05	82	(33-275)	0.62	12

*95% fiducial limits; **resistance factor = LC_{50} ALM-2 / LC_{50} SUD-S

Systemic bioassay

The systemic bioassay has been described recently as a possible method for monitoring the efficacy of imidacloprid against field strains of *B. tabaci*, because it mimicks the possible field exposure to this insecticide best (Elbert *et al.*, 1996; Cahill *et al.*, 1996b). Table 3 shows the results with imidacloprid, expressed as LC_{50}-values. As with the leaf-dip bioassay, the systemic

test revealed ALM-2 as the least susceptible strain against imidacloprid. The resulting resistance factors were quite low again. The CAL-1 strain as a representative of a typical B-strain behaves as susceptible as the reference SUD-S. The results on the Spanish strains were in contrast to those published very recently by Cahill et al. (1996b), but both M Cahill and authors are working closely together to find out the reasons for the observed differences. Some points need to be addressed, e.g. different cotton varieties and leaf sizes were used in the bioassays. Cahill et al. (in press) found no resistance to imidacloprid in whitefly strains from Australia, Israel, Netherlands, Pakistan and USA using the systemic bioassay, but a moderate level of tolerance in some strains of B. tabaci from Spain. The systemic bioassay should be the first choice monitoring bioassay, because it is reliable, quick and simple.

Table 3. Efficacy of imidacloprid against B. tabaci in a systemic bioassay

Strain	Imidacloprid $LC_{50(48h)}$ (ppm)	*95% FL	Slope	**RF
SUD-S	7.0	(5.3-9.3)	1.00	1
ALM-2	35	(20-56)	1.23	5
LMPA-2	17	(11-29)	1.12	2
CAL-1	7.3	(3.2-16)	1.10	1

*95% fiducial limits; **resistance factor = LC_{50} other strains / LC_{50} strain SUD-S

Egg/larval-dip bioassay

This bioassay was primarily performed to get some information on the activity of imidacloprid on the different developmental stages of B. tabaci. The bioassays were conducted with SUD-S and ALM-2, the least susceptible Spanish biotype. Table 4 shows the good activity of imidacloprid against all developmental stages, with the highest susceptibility recorded on eggs. Compared to SUD-S all developmental stages of strain ALM-2 are somewhat less susceptible (3-5 times) to imidacloprid, though not highly significant in all cases. The results obtained with buprofezin, an insect growth regulator, on 2nd/3rd instar of B. tabaci were approximately in the same range as those shown for imidacloprid. LC_{50}-values for SUD-S and ALM-2 were 9.7 ppm and 46 ppm, respectively. However, as expected from another already published study (Cahill et al., 1996b) the Spanish strain ALM-2 showed a 5-fold resistance to buprofezin, though the LC_{50}-values in our study were somewhat higher.

Table 4. Efficacy of imidacloprid on different developmental stages of B. tabaci

Developmental Stage	SUD-S LC_{50}, (ppm)	*95% FL	Slope	ALM-2 LC_{50}, (ppm)	*95% FL	Slope	**RF
Eggs	1.1	(0.53-2.0)	1.92	3.2	(1.0-10)	1.71	3
1st instar	13	(4.3-23)	2.19	59	(46-73)	2.54	5
2nd/3rd instar	4.7	(3.3-6.3)	1.65	24	(8.4-45)	2.16	5
4th instar	7.0	(0.99-16)	1.30	29	(21-39)	1.46	4

*95% fiducial limits; **resistance factor = LC_{50} ALM-2 / LC_{50} SUD-S

Sachet-test

The lowest LC_{50}-values for all strains were found when testing the oral efficacy of imidacloprid using artificial double membranes, incorporating imidacloprid in aqueous sucrose solutions. The resulting resistance factors for the field strains of *B. tabaci* were as low as with all other bioassay systems described above (Table 5). SUD-S and CAL-1 showed the highest susceptibility. The suitability of the sachet-test as a possible monitoring tool has been confirmed using monocrotophos (Table 6). Biochemical examinations revealed that all strains except SUD-S have an insensitive acetylcholinesterase (results not shown), and the expected high resistance factors against monocrotophos using the artificial feeding bioassay, which was also successfully used in resistance studies with aphids (Nauen *et al.*, 1996), were found.

Table 5. Efficacy of imidacloprid against *B. tabaci* using the sachet-test

Strain	Imidacloprid $LC_{50(48h)}$ (ppm)	*95% FL	Slope	**RF
SUD-S	0.24	(0.064-0.59)	1.50	1
ALM-2	0.44	(0.30-0.62)	0.98	2
LMPA-2	1.27	(0.33-4.1)	1.38	5
CAL-1	0.26	(0.12-0.56)	1.78	1

*95% fiducial limits; **resistance factor = LC_{50} other strains / LC_{50} strain SUD-S

Table 6. Efficacy of monocrotophos against *B. tabaci* using the sachet-test

Strain	Monocrotophos $LC_{50(48h)}$ (ppm)	*95% FL	Slope	**RF
SUD-S	7.9	(4.5-15)	3.11	1
ALM-2	292	(195-438)	1.06	37
LMPA-2	501	(269-791)	3.87	63
CAL-1	119	(30-547)	1.67	15

*95% fiducial limits; **resistance factor = LC_{50} other strains / LC_{50} strain SUD-S

Field trials with imidacloprid in southern Spain

IRAC (Insecticide Resistance Action Committee) defined resistance by establishing four criteria. One of them says: The recommended dosages fail to suppress the pest population below an economic threshold. Therefore the efficacy of imidacloprid against *B. tabaci* has been monitored for several years in a region, where the product has been used regularly. Intensive vegetable grown areas in Almeria and Murcia have been selected for this examination. In Almeria for example 19,000 ha of pepper (7,000 ha), tomato, (6,000 ha), beans and zucchini (4,000 ha) and pepino (2,000 ha) are grown under plastic in two crop cycles per year. In addition, tomato yellow leaf curl virus (TYLCV) and cucumber yellow virus (CYV) are transmitted frequently by *B. tabaci* and *Trialeurodes vaporariorum* respectively. Also,

resistance against various insecticides has developed and is widespread in this region (Cahill *et al.*, 1996a and b). Insecticides are therefore frequently used. After two applications, excellent control of *B. tabaci* has been demonstrated in all trials between 1988 and 1996. The efficacy ranged between 90 and 100 % Abbott. Even after continuous commercial use from 1992 onwards, a high level of activity (93 to 99%) was confirmed in the years 1994 to 1996. From these results, no loss of activity of imidacloprid against the whitefly *B. tabaci* could be detected under practical conditions (Table 7).

Table 7. Efficacy of imidacloprid 200 SL against *B. tabaci* in southern Spain from 1988 - 1996

Year	1988*	1989*	1991	1994	1995	1995	1996
Trial no.	102a/88	27/89	48/91	82/94	08/95	10/95	20011/96
Crop	Pepper	Bean	Bean	Pepper	Pepper	Pepper	Pepper
Region	Murcia	Almeria	Almeria	Murcia	Murcia	Almeria	Almeria
Site	Ramonete	Palomares	San Augustin	Dolores	San Pedro	La Mojonera	El Ejido
Mortality (% Abbott)	90	97	100	93	98	97	94

* Mixed populations *B. tabaci / T. vaporariorum*

Being aware of the high capability of whiteflies to develop resistance, general guidelines for a resistance management for chloronicotinyl insecticides have been defined (Elbert *et al.*, 1996). Currently, a two year project is underway with Cahill and Devonshire, IACR Rothamsted to design a resistance management strategy for *B. tabaci* in Southern Spain.

ACKNOWLEDGEMENTS

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LONG-TERM STABILITY IN THE FREQUENCY OF INSECTICIDE RESISTANCE IN THE PEACH-POTATO APHID, *MYZUS PERSICAE*, IN ENGLAND

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ABSTRACT

Insecticide resistance in *Myzus persicae* in four field crops has been monitored for 8 years and seasonal variation in resistant frequencies occurred throughout this period. There has been no increase in the frequency of the most resistant R2 and R3 variants despite continuing insecticide application. The frequency of the R1 variant has apparently increased in only one crop. The stability of this resistance appears to be maintained by a balance between insecticide selection for the resistant variants during the summer and selection against them during the winter.

INTRODUCTION

Myzus persicae (the peach-potato aphid) is a major pest of a wide range of economically important field and glasshouse crops, primarily as a vector of virus diseases. Susceptible crops are usually treated with insecticide as a matter of routine with the result that there can be high selection pressure for insecticide resistance which has consequences for the control of both this aphid and the viruses it transmits.

The first signs of insecticide resistance in *M. persicae* from the USA in the 1950s were confirmed in 1963 by Georghiou (1963) and then in the UK in 1965, in glasshouse populations, by Wyatt (1965). The differential susceptibilities of field populations were demonstrated about ten years later (Sawicki *et al.*,1978) when aphids were individually classified as susceptible (S), moderately resistant (R1), and strongly resistant (R2). Later Devonshire & Sawicki (1979) added an additional category of extremely resistant (R3) aphids. R1 aphids are controlled with some organophosphates, but R2 aphids are best controlled with carbamates. R3 aphids are not well controlled by any product approved for use on outdoor crops.

Due to concerns about the spread of resistance in *M. persicae*, and in particular the most resistant R2 and R3 variants which could be difficult to control in the field if they became predominant, a number of surveys of resistance in *M. persicae* have been carried out (Sawicki *et al.*, 1978, Furk, 1986, and Hockland *et al.*, 1992). A survey to monitor resistance in *M.*

persicae has collected data since 1988 and it seems an opportune time to review the progress of the resistance, thirty years after it was first confirmed in the UK.

MATERIALS AND METHODS

Samples of *M. persicae* were collected from 1988 to 1995 from oilseed rape, vegetable brassicas, potatoes and sugar beet, and also from glasshouses from 1992, on a generally opportunist basis by ADAS entomologists and sent to CSL for testing. At some sites, CSL staff made monthly visits to collect aphids. On receipt, individual mature aphids were placed in one of 84 of the 96 wells of a microplate containing 50µl of phosphate-buffered saline (PBS) with Tween and frozen at -20°C before analysis up to six weeks later. The remaining wells were used for standards. Immature aphids were cultured on single leaves of cabbage (*Brassica oleracea*) kept fresh in "florists" sponge inside Blackman boxes and frozen when mature. Aphids were tested for insecticide resistance using the immunoassay technique of Devonshire *et al.* (1986). Aphids were classified into one of four broad groupings, S, R1, R2, and R3, according to their esterase activity relative to the standard clones maintained in the laboratory.

RESULTS

Between 1988 and 1995, a total of 22,121 aphids were collected from the four field crops. Aphids were obtained from oilseed rape and vegetable brassicas throughout the year, but the majority (81%) from potatoes were collected between May and September and from sugar beet between March and June (80%). More than half were of the moderately resistant (R1) variant and more than a quarter were susceptible (S). The results for each crop and each year are shown in Table 1.

The eight year totals for each crop show that the frequencies of the susceptible and resistant variants differ between the crops. The fewest susceptible variants were found on oilseed rape and vegetable brassicas; oilseed rape had the greatest proportion of the R2 and R3 variants. For all the crops, the frequencies differ from year to year and there is significant heterogeneity within each data set, but for oilseed rape, potatoes and sugar beet there is no discernible trend to the changes of frequency. The aphids from vegetable brassicas, however, do show a general increase in the frequency of the R1 variant.

Between 1992 and 1995, a further 1607 *M. persicae* were collected from a variety of plants, including weeds, growing under glass. Of these aphids 29% were R3 variant, 51% were R2, 18% were R1 and only 2% were susceptible. These results are almost the inverse of those from the field crops which, for the same period, were 1% R3, 11% R2, 61% R1 and 27% S.

The frequency of the susceptible and resistant variants in each of the four seasons is shown in Table 2. The results are only given for oilseed rape and vegetable brassicas, those crops from which aphids could be collected throughout the year. For both crops there is a large change in frequency when the spring (April-June) and autumn data (October-December) are compared.

Table 1. The frequency of S, R1, R2 and R3 individuals of *Myzus persicae* collected from oilseed rape, vegetable brassicas, potatoes and sugar beet each year 1988-1995.

	S	R1	R2	R3	Total (numbers)
Potatoes					
1988	0.35	0.49	0.16	0.01	2144
1989	0.46	0.52	0.02	0.01	2236
1990	0.23	0.7	0.07	<0.01	903
1991	0.03	0.35	0.39	0.23	57
1992	0.35	0.49	0.15	0.01	185
1993	0.32	0.52	0.12	0.04	584
1994	0.59	0.41	0	0	32
1995	0.47	0.51	0.03	0	390
All years	**0.37**	**0.53**	**0.09**	**0.01**	**6531**
Oilseed rape					
1988	0.33	0.51	0.15	0.01	946
1989	0.24	0.60	0.15	0.01	3460
1990	0.20	0.52	0.27	0.02	2556
1991	0.13	0.49	0.29	0.09	1083
1992	0.22	0.59	0.18	0.01	1678
1993	0.37	0.54	0.08	<0.01	951
1994	0.28	0.44	0.28	0	36
1995	0.57	0.42	<0.01	0	113
All years	**0.24**	**0.55**	**0.19**	**0.02**	**10823**
Vegetable brassicas					
1988	0.69	0.20	0.10	0.01	135
1989	0.47	0.48	0.05	0	533
1990	0.24	0.60	0.16	<0.01	572
1991	0.15	0.68	0.11	0.06	469
1992	0.05	0.81	0.11	0.03	684
1993	0.11	0.88	0.01	0	281
1994	0.76	0.21	0.03	0	160
1995	0.10	0.76	0.14	0	494
All years	**0.24**	**0.65**	**0.10**	**0.02**	**3328**
Sugar beet					
1988	0.06	0.39	0.55	0	108
1989	0.29	0.71	<0.01	0	687
1990	0.45	0.47	0.09	0	294
1991	0.25	0.44	0.27	0.05	105
1992	0	1.00	0	0	1
1993	0.10	0.83	0.07	0	98
1994	0.84	0.16	0	0	19
1995	0.27	0.70	0.03	0	98
All years	**0.29**	**0.61**	**0.09**	**<0.01**	**1410**
All crops, all years	**0.28**	**0.56**	**0.14**	**0.01**	**22122**

Table 2 Seasonal frequencies of S, R1, R2 and R3 *M. persicae* collected between 1988 and 1995

Crop	Months	S	R1	R2	R3	Number tested
Oilseed Rape	January-March	0.327	0.648	0.023	0.002	2591
	April-June	0.368	0.582	0.045	0.005	1007
	July-September	0.168	0.454	0.342	0.035	2584
	October-December	0.203	0.550	0.227	0.020	4674
Vegetable brassicas	January-March	0.260	0.680	0.040	0.020	696
	April-June	0.376	0.602	0.018	0.004	1121
	July-September	0.166	0.632	0.196	0.006	669
	October-December	0.086	0.686	0.178	0.050	596

DISCUSSION

Two important conclusions may be drawn from these results; first, in three of the four crops the frequencies of resistant varieties have not increased during the study period and second, there are clear seasonal differences in the frequency of the resistant and susceptible variants. These two findings may well be linked and have important consequences for pest management.

The data in Table 1 show that the frequency of resistance in *M. persicae* collected from oilseed rape, potatoes and sugar beet has not increased during the eight years. If anything the frequency of the susceptible variant is now higher than in 1988. This is not true for vegetable brassicas where there has been a steady and significant decrease in the frequency of the susceptible variant. This trend would be more marked if the results for 1994, when aphids were only collected in the first half of the year, were excluded. For none of these crops is there any evidence of an increase in the frequency of R2 and R3 variants. Thus the variants most likely to give rise to control failures in the field remain rare and the concern that the resistant variants are increasing is not borne out. There are significant fluctuations from year to year but with the exception of the results from vegetable brassicas, these changes are not sustained. In fact, the overall situation remains similar to that found by Furk (1986) who sampled the same crops from 1980-84. His data are compared in Table 3 with the equivalent data for 1992-95. It can be seen that for the *M. persicae* collected from potatoes and sugar beet there has been no change in the frequencies of the variants comparing 1980-84 with 1992-95. For oilseed rape, the frequency of susceptible aphids has increased and strangely this has been accompanied by an increase in the frequency of R2 + R3; the increases were accompanied by a decrease in the frequency of R1. The increase in the frequency of the R1 variant from vegetable brassicas, already noted for 1988-95, is also apparent here and on this crop there is also an increase in the frequency of R2 + R3. Overall, comparing the two sets of data, there is an increase in the frequency of R2 + R3 and a decrease in the frequency of R1.

Furk (1986) remarked that his data were very similar to those collected in 1976 by Sawicki *et al.* (1978) who found that 69% of the aphids they collected from potatoes were resistant, and this compares with 63% for the current study. Thus it would seem that, with the exception of aphids from vegetable brassicas, there has been no substantial change in the frequencies of resistant variants for many years. However, some caution must be exercised in making these comparisons with earlier years, as both Sawicki *et al.* (1978) and Furk (1986) used the electrophoretic method (Devonshire, 1975) of detecting resistance in *M. persicae,* whereas for the current study we have used the immunoassay method (Devonshire *et al.*, 1986). Some

differences in classifying the resistant variants are likely, but as the great majority are of the R1 variant we do not believe that this is a significant problem, given the large numbers tested and the frequencies found.

Table 3. The frequency of S, R1 and R2+R3 *Myzus persicae* collected from four field crops in England and Wales from 1980 to 1984 (data from Furk, 1986) with the equivalent data from 1992 to 1995 for comparison. In the earlier set of data the R2 and R3 variants were not separated..

Crop		S	R1	R2+R3	number tested
Potatoes	1980-84	0.36	0.54	0.09	1324
	1992-95	0.38	0.51	0.10	1191
Oilseed Rape	1980-84	0.13	0.84	0.03	724
	1992-95	0.29	0.56	0.15	2778
Vegetable brassicas	1980-84	0.29	0.69	0.02	447
	1992-95	0.15	0.75	0.10	1619
Sugar beet	1980-84	0.23	0.74	0.03	148
	1992-95	0.24	0.71	0.05	216
All	1980-84	0.28	0.66	0.06	2603
	1992-95	0.27	0.61	0.12	5804

The overall stability of this resistance, in spite of continuing insecticide usage, requires some explanation. A possible explanation arises from the seasonal variation in the frequency of the variants as shown in Table 2. Furk *et al.*, (1990) showed that during the winters of 1988/89 and 1989/90 the frequency of the R2 and R3 variants of *M. persicae* decreased throughout the winter so that these variants were virtually absent from January onwards. These data, together with those in Table 2, confirm this decrease in the R2 and R3 variants and show that it has been repeated over a number of years and on more than one crop. These seasonal fluctuations in frequencies of the S, R2 and R3 variants may be attributed to the effect of insecticide selection during the summer being countered by poor overwinter survival of the R2 and R3 variants, which has recently been demonstrated by Foster *et al.* (1996).

The situation in *M. persicae* could therefore be analogous to a stable polymorphism where two or more forms are maintained in a population by opposing selective forces, in this case insecticide pressure and overwintering success. Such polymorphisms are rare but Haldane & Jayakar (1963) showed that they can be maintained providing that the opposing selective forces are large. The selective values which would maintain stability would fall within a narrow range and changes in these values could produce rapid shifts in frequency in favour of either the susceptible or the resistant variants. It follows that any change in current pest management procedures could upset this balance. The high levels of the R2 and R3 variants in *M. persicae* from glasshouses show the potential for this resistance to develop when one of the selective pressures, overwintering, is eliminated. This explanation can only be applied to the failure of the R2 and R3 variants to increase. For the R1 variants, which appear not to be at a disadvantage during the winter, some other factor must be preventing their increase.

This survey is based on the incidence of esterase-based resistance which is the predominant mechanism conferring broad cross-resistance in this species world-wide. However, another mechanism based on carbamate-insensitive acetylcholinesterase has been identified from sites around the world, including northern Europe (Moores *et al.*, 1994), and at very low frequency

in the UK. In view of its adverse effect on the performance of pirimicarb, which is used for controlling *M. persicae* with esterase-based resistance, it will be important for future resistance monitoring programmes to determine the incidence of this mechanism.

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TWO FORMS OF INSECTICIDE-INSENSITIVE ACETYLCHOLINESTERASE IN *APHIS GOSSYPII*

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ABSTRACT

Levels of resistance and acetylcholinesterase (AChE) sensitivity to pirimicarb and organophosphorus insecticides were examined in three clones of *Aphis gossypii* and in a non-clonal culture derived from a sexually-reproducing population in China. Two insensitive AChE variants were identified with differing levels of insensitivity to these chemicals. A diagnostic procedure for identifying these variants in individual aphids, whether homozygous or heterozygous, and correlations between AChE insensitivity and insecticide resistance determined by bioassays are presented, and discussed.

INTRODUCTION

The cotton aphid, *Aphis gossypii*, is a major agricultural pest, and heavy reliance is placed on insecticides for its control. Resistance has been reported from around the world, with enhanced esterase activity being most commonly implicated as a mechanism conferring protection against carbamate, organophosphorus (OP) and pyrethroid insecticides (Takada & Murakami, 1988 and Hama & Hosada, 1988). An insensitive acetylcholinesterase (AChE) has also been reported that confers strong resistance to pirimicarb (Gubran *et al*, 1992 and Silver *et al*, 1995). Work to characterise AChE insensitivity to a wider range of insecticides including pirimicarb, and to correlate insensitivity profiles with levels of resistance determined from leaf-dip bioassays against adults are reported. Data for a sexually-reproducing *A.gossypii* strain from China that provide further information on the nature and inheritance of insecticide-insensitive AChE variants are also presented.

MATERIALS AND METHODS

Aphids

Clone 171B was isolated from a population collected from the UK in 1981 (Furk & Vedjhi, 1990) and has since been maintained as a reference susceptible clone. Clones 968E and 1081K, each also derived from a single female, were collected from cotton in Greece in 1991 and Zimbabwe in 1992, respectively. The China strain originated from

Nanjing in 1996 from eggs resulting from sexual reproduction on *Hibiscus* (species not determined). All were maintained as parthenogenic cultures in the laboratory in the absence of insecticide selection on excised cotton leaves.

AChE monitoring in single aphids

AChE diagnosis was performed as described previously (Moores *et al*, 1988). Individual aphids were homogenised in 200 µl 0.1M phosphate buffer (pH 7.5, containing 0.1% Triton X-100) in separate wells of a microplate, and left for 30 min at 4^0 C to allow full solubilisation of the AChE. Three replicates of homogenate (50 µl) were added to a fresh microplate, and buffer (50 µl) and 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) in 100 µl of buffer added. Assays were started with the addition of ATChI in buffer (100 µl) with and without a final diagnostic concentration of pirimicarb (50 µM) or demeton-S-methyl (200 µM). Final concentrations in each of the assays were 0.5 mM substrate and 15 µM DTNB. The reactions were monitored for 20 mins using a Molecular Devices Thermomax microplate reader. Linear regressions were fitted to the AChE activity over this time, and the results expressed as percentage activity remaining compared to the uninhibited replicate. This compensated for any differences in AChE activity in individuals within or between clones and strains. Artificial heterozygotes were created by homogenising two aphids, one of each genotype, in the same well, and adding three replicates of homogenate (25 µl) to wells, together with an extra 25 µl buffer to maintain the volume.

 Leaf-dip bioassay   Cotton (*Gossypium hirsutum* cv. Deltapine 16) leaf discs (35 mm diameter) were dipped in aqueous solutions of formulated insecticide, placed upside down on an agar bed (25 mm in depth) in disposible plastic containers (30 mm high) and allowed to air-dry. Apterous adults of the required clone (10 per container) were placed on the treated leaf surface and confined by a ring of fluon applied to the exposed lip of the container. Leaf discs dipped in water plus Agral were used as controls. Bioassay containers were stored upright, without lids, in a constant environment facilty at 25°C under continuous room lighting.

Dose-response bioassays were conducted using three replicate batches of aphids (i.e. 30 insects) at each of five insecticide concentrations identified from range-finder tests. Tests were scored after 48 h continuous exposure to insecticide residues. Adults failing to exhibit repetitive (ie. non-reflex) movement of more than one leg (after gentle prodding if necessary) were scored as dead. Results for at least two independent bioassays were pooled and subjected to probit analysis using the POLO computer program (LeOra Software, Berkeley, California). Resistance ratios were calculated by dividing LC_{50} values computed for resistant aphid cultures by the corresponding LC_{50} for the susceptible 171B reference clone.

RESULTS AND DISCUSSION

Resistance levels

1081K adults showed strong (>70-fold) resistance to pirimicarb in leaf-dip bioassays, but much lower (<20-fold) resistance to the OPs, demeton-S-methyl and omethoate (Table 1).
968E aphids exhibited enhanced resistance to all three chemicals. Resistance ratios for OP's were 7- to 10-fold higher than corresponding figures for 1081K. For clonal cultures such variations cannot be attributed to different frequencies of the same resistance allele. It therefore implicates two contrasting modes of resistance conferred by the same underlying mechanism and/or at least one additional mechanism contributing to elevated resistance levels in 968E.

Table 1. LC_{50} (95% confidence limits) and resistance ratios(RR) of standard clones to insecticides

Clone Insecticide	171B		1081K		RR	968E		RR
Pirimicarb	53	(45-62)	3900	(3300-4600)	74	>20000		>380
Triazamate	0.65	(0.43-0.88)	37	(33-43)	57	20	(17-24)	30
Demeton -S-methyl	2.3	(1.7-2.9)	41	(28-62)	18	410	(320-490)	180
Omethoate	1.6	(1.5-1.8)	14	(8.7-20)	9	95	(79-110)	60

AChE diagnosis

Bivariate plots of AChE activity remaining during the incubation of homogenates of individual aphids with pirimicarb and demeton-S-methyl identified distinct levels of AChE insensitivity in clones 1081K and 968E (Fig 1). A concentration of 50 μM pirimicarb separated the fully sensitive enzyme found in 171B from both insensitive variants, and 200 μM demeton-S-methyl distinguished between the enzymes present in the resistant clones. There are two possible interpretations of these results. Either the difference in AChE insensitivity between 1081K and 968E was due to the same mutant AChE allele present in homozygous condition in 968E and heterozygous combination with the sensitive variant in 1081K, as has been found in *Myzus persicae* (Moores *et al*, 1994), or the two clones contained distinct allelic variants of insensitive AChE. To investigate this, artificial heterozygotes were generated by homogenising a single 171B aphid with one from either 1081K or 968E. AChE inhibition analysis then gave a discrete cluster of points in the bivariate plot intermediate to the two putative homozygote 'parents', but displaced due to differences in catalytic activity, towards the

'parent' possessing the more active enzyme. This supports the hypothesis that each clone possessed a biochemically-distinct AChE variant, the greater insensitivity of the 968E enzyme to OPs being reflected in the higher OP resistance of the clone in bioassay (Table 1).

Additional evidence for multiple AChE alleles came from applying this assay to the China strain. This revealed the presence of the sensitive and both insensitive AChE variants, plus clusters of points indicative of heterozygotes resulting from sexual reproduction (Fig 2).

Fig. 1. Bivariate plot of *A.gossypii* clones., showing the percentage of AChE activity remaining during inhibition with pirimicarb and demeton-S-methyl.

    Filled squares represent AChE from the standard laboratory clones, 171B, 1081K and 968E. Empty squares represent AChE from mixed homogenates of those clones.
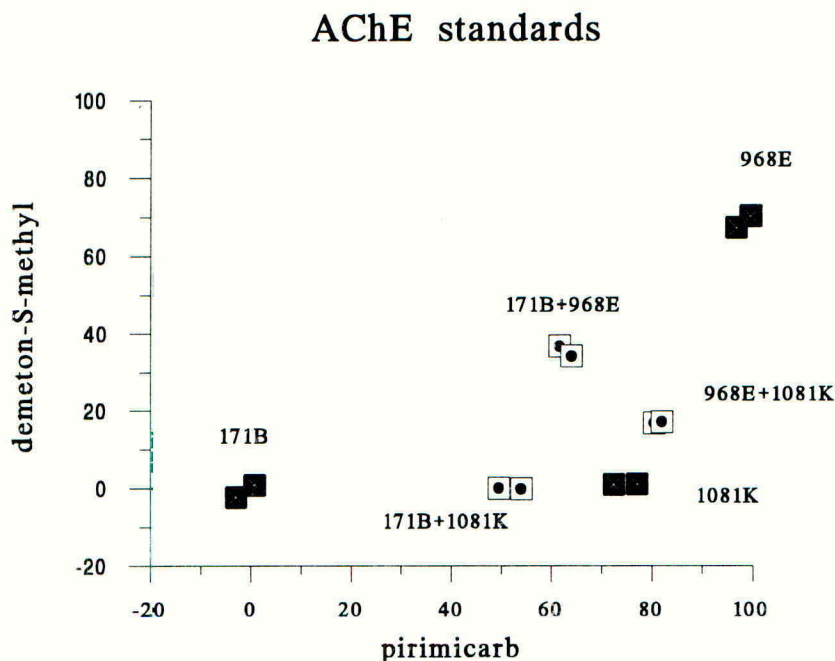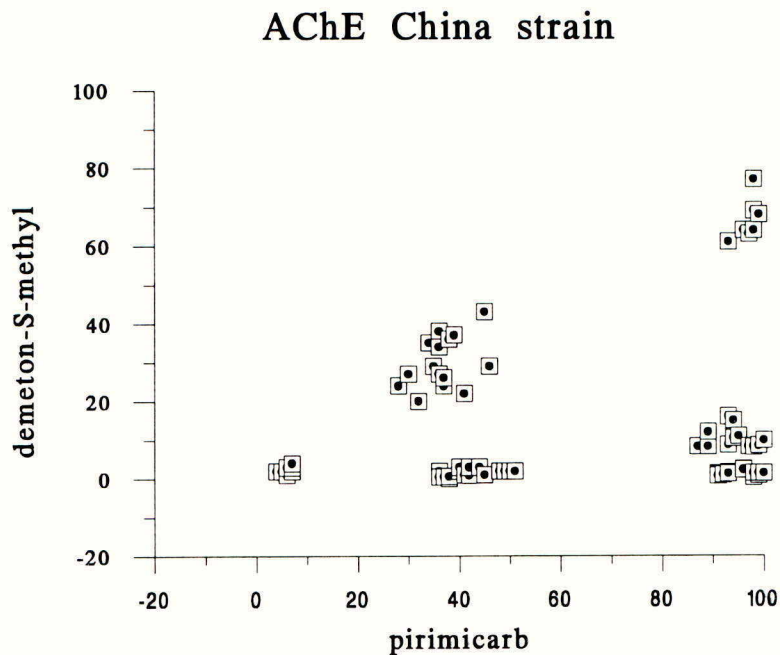

AChE standards

Fig.2.Bivariate plot showing distribution of the China strain, showing the percentage of AChE activity remaining during inhibition with pirimicarb and demeton-S-methyl. Each square represents an individual aphid.

AChE China strain

CONCLUSIONS

Although the role AChE insensitivity in conferring resistance to pirimicarb in *A.gossypii* is well established (Gubran *et al*, 1992 and Silver *et al*, 1995, the implications of the mechanism for control with OPs have remained less clearcut. These findings demonstrate that the prospects for managing pirimicarb-resistant aphids with OPs depend critically on the type and frequency of insensitive AChE enzyme present. Populations possessing the

968E variant are far less likely to be controlled satisfactorily than the ones with the 1081K variant, although the relationship between bioassay results and the performance of chemicals in the field has yet to be investigated

A microplate assay incorporating diagnostic concentrations of pirimicarb and demeton-S-methyl offers exciting scope for ditinguishing rapidly between the two known insensitive variants, and hence anticipating the resistance characteristics of field populations. However, AChE may not be the only resistance mechanism present, and so care must be taken in exploiting such findings to decrease control tactics in the field. The power of the assay is reinforced by its ability to distinguish not only allelic forms of AChE but also homozygotes from heterozygotes (Moores *et al*, 1994). It thus provides a powerful tool for analysing AChE resistance gene build-up in populations under selection in both the field and the laboratory.

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USE OF NEW ALKYNYL SYNERGISTS TO COUNTER INSECTICIDE RESISTANCE

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ABSTRACT

New alkynyl synergists were synthesised and tested in the laboratory by co-administration with different insecticides including representatives of organochlorines, carbamates, organophosphorous, pyrethroids and macrocyclic lactons. The synergistic potency of these chemicals was simultaneously determined on a susceptible WHO/SRS, two laboratory-selected resistant (pyrethroid-resistant CHXSEL and carbamate-resistant CARBSEL) and a field collected multiple resistant (MD-IX) house fly (*Musca domestica*) strains. Spectrum of synergistic action and the cross-resistance patterns proved to be characteristic of strain.

A laboratory selection experiment on housefly using carbofuran or a new synergist, MB-279+carbofuran as selecting agents showed that resistance did not evolve at all to the mixture compared to the selection which used carbofuran alone. While the carbofuran selected group (CARBSEL) showed 918 and 600 resistance ratios at F_8 generation in the female and male flies respectively, the group selected with the mixture (CS279) collapsed at F_4. Moreover, high suppression in adult emergence from pupae was obtained in the fly group selected jointly with carbofuran+synergist, but not in the group selected only with carbofuran.

INTRODUCTION

Insecticide resistance is currently a worldwide problem and has become one of the most important obstacles to the efficient control of insect pests (Georghiou 1990). Several management tactics are available to prevent, delay or suppress the resistance evolution (Leeper *et al.*, 1986). Synergists, which are non-toxic alone but enhance the potency of insecticides by blocking specific detoxification enzymes, have been considered as a promising alternative tool for impeding the development of resistance (Henessy, 1971). Co-application of synergists with insecticides is believed to be efficient particularly when a single resistance mechanism is involved (Roush & Daly, 1990). Despite the availability of dozens of potent experimental synergists, their field application has been restricted by several factors, including cost-effectiveness, toxicological and environmental hazards, application difficulties and uncertainties in registration (B-Bernard & Philogene, 1993).

Cytochrome P-450-dependent polysubstrate mono-oxygenases (PSMOs) play a principal role in metabolism of numerous insecticides (Agosin, 1985), and, therefore are ideal targets for development of synergists. This is supported by the fact that the two most important commercial synergists, piperonyl butoxide (PBO) and ENT-8148 (MGK-264), are inhibitors of PSMOs. Recently, a new synergist family with several highly potent members, including MB-599 (proposed common name verbutin) was discovered (Árvai *et al.*, 1995; Székely *et*

al. 1996). Comparative structure-activity relationship studies (Bertók, unpublished) and 3D docking calculation to binding site of enzyme (Keserû, unpublished) suggest that alkynyl side-chain of six atoms is preferable for high synergist activity of the analogues and that they presumably act as inhibitors of PSMOs.

The aim of the studies presented here was to investigate the suitability of this type of synergist for resistance-countering. To this end, the effect of the carbofuran + synergist mixture on progression of resistance was compared with the carbofuran alone. Synergistic potency of some analogues on several insecticides was simultaneously assessed on susceptible WHO/SRS, two laboratory-selected resistant (pyrethroid-resistant CHXSEL and carbamate-resistant CARBSEL) and a field collected multiple resistant (MD-IX) housefly strains.

MATERIALS AND METHODS

Chemicals

MB-113 (4-(2-methyl)-quinoline prop-2-ynyl ether), MB-279 ((1-naphtyl)-methyl but-2-ynyl ether), and MB-599 (1-(3,4-dimethoxyphenyl)-ethyl but-2-ynyl ether, proposed common name: verbutin)) were synthesised by the chemists of Chinoin. Piperonyl butoxide (PBO, Fluka AG) and ENT-8184 (N-(2-ethylhexyl)-8,9,10-trinorborn-5-ene-2,3-dicarboximide, MGK-264, Bábolna Bio) were used as reference synergists. Aldicarb, aminocarb, bendiocarb, carbaryl, carbofuran, dioxacarb, endosulfan, isoprocarb, fenobucarb, methomyl, pirimicarb, propoxur, phosmet, quinalphos, tetrachlorvinphos and thiodicarb were obtained from Riedel-de Haën in analytical grade. Permethrin (*cis/trans* ratio:2/3, 96.7 %) and beta-cypermethrin (mixture of (S)(1R)*cis*/(R)(1S)*cis*/(S)(1R)*trans*/(R)(1S)*trans* cypermethrin isomers, ratio: 2/2/3/3, > 99 %) were manufactured in Chinoin. Ivermectin used as commercial formulation (Ivomec, MSD).

Housefly strains

The following housefly (*Musca domestica*) strains were used. WHO/SRS strain, obtained originally from Prof. Milani, was used as the susceptible reference. The CHXSEL strain was maintained under consecutive and constant selection pressure with beta-cypermethrin (Pap & Tóth, 1995). The multi-resistant MD-IX strain, which was collected from a pig farm during a previous resistance survey in Hungary (Pap & Farkas, 1994) and has been subjected to selection with beta-cypermethrin to achive genetic homogeneity. A sub-strain of the original WHO/SRS strain was used in the selection study.

Bioassay

Dose-mortality tests with serial dilution of insecticides in cellosolve were carried out by topical application of 0.2 µl/fly test solution (Pap & Farkas 1994). Mortality was recorded 24 h after treatment. Four to seven doses and at least two replicates were used for each bioassay. The pooled mortality data were subjected to probit-analysis. Synergists were applied at a constant dose (1 µg/fly) with insecticides in test solutions. This dose of synergists applied alone did not cause mortality of treated flies of either strains. Synergist

ratio (SR) was given as a quotient of the LD_{50} values of insecticide administered alone and with the synergist.

Selection

About 1000 flies of each sex of the selected WHO/SRS sub-group, were treated with carbofuran (sub-strain CARBSEL) or carbofuran+MB-279 (sub-strain CS279) by topical application for nine consecutive generations. The synergist was administered at 1 μg/fly. Mortality was recorded after 24 h treatment and the survivors were held in a rearing cage as parents of the next generation. Details of the selection procedure are summarised in Table 1. The resistance ratio (RR) was calculated as the quotient of the LD_{50} of selected generation and the LD_{50} of the parental strain.

Table 1. History of selection of the CARBSEL (carbofuran selected) and CS279
(carbofuran+MB-279 slected) housefly sub-strains

Selected generation	Selecting dose (μg/fly)		Number of flies selected		Mortality %	
	Female	Male	Female	Male	Female	Male
CARBSEL sub-strain						
Parental	0.2	0.1	800	660	55	80
1	0.4	0.1	1280	1280	46	38
2	1.6	0.4	1000	1000	74	81
3	3.2	0.8	1020	1020	39	65
4	8.0	2.0	1140	1220	71	80
5	10.0	5.0	1240	1320	57	82
6	20.0	10.0	1100	1380	59	83
7	40.0	20.0	1140	1180	90	93
8	60.0	30.0	1020	1080	91	86
9	75.0	40.0	680	740	70	95
CS279 sub-strain						
Parental	0.01	0.005	1240	1320	41	34
1	0.02	0.01	740	700	58	48
2	0.08	0.04	1040	1000	86	95
3	0.16	0.08	1000	1000	96	95
4	0.20	0.10	220	220	97	84
5[a]	-	-	-	-	-	-
6[a]	-	-	-	-	-	-
7	0.20	0.10	80	100	98	100

[a] Generation not treated with carbofuran+MB-279 due to low number of progeny produced by surviving flies.

RESULTS AND DISCUSSION

Resistance to carbofuran rose rapidly in CARBSEL sub-strain. Increasing selection pressure from the dose of 0.2 and 0.1 μg/fly to 75 and 40 μg/fly resulted in a resistance ratios of 918

and 600 in female and male flies respectively by the F_9 generation (Table 2). In contrast, no resistance was evolved at all in CS279 sub-strain using the synergistic mixture of carbofuran and MB-279 as the selecting agent. The apparent lack of increase in resistance ratio of CS279 substrain suggests the high potency of MB-279 in prevention or in reducing the rate of carbofuran resistance evolution. Moreover, due to decreased number of progeny produced by surviving flies and their dramatically low fecundity, the CS279 sub-strain had collapsed by the F_7 generation.

Table 2. Resistance evolution in CARBSEL (carbofuran selected) and CS279 (carbofuran+MB-279) housefly sub-strain.

Selected Generation	CARBSEL sub-strain						CS279 sub-strain					
	Female			Male			Female			Male		
	LD_{50}[a]	Slope	RR[b]	LD_{50}[a]	Slope	RR	LD_{50}[c]	Slop	RR	LD_{50}[c]	Slope	RR
P	0.17	1.97	-	0.04	1.41	-	8.7	2.07	-	7.3	3.9	-
1	0.45	1.88	3	0.16	1.32	4	21.6	2.80	3	7.5	2.90	1
2	0.69	1.21	4	0.11	1.73	3	21.5	4.12	3	4.9[d]	3.16	1
3	1.47	1.44	9	0.27	1.45	8	12.6[d]	2.30	2	4.6	1.56	1
4	1.57	1.38	9	0.84	1.50	21	-[e]			-[e]		
5	16.0	1.37	94	3.27	1.25	82	-[e],[f]			-[e],[f]		
6	16.2	1.31	95	3.18	1.90	80	-[e],[f]			-[e],[f]		
7	10.7	0.75	63	3.86	0.99	97	-[g]			-[g]		
8	41.3	2.79	243	20.8	2.88	130	-			-		
9	156.0	1.17	918	24.0	2.38	600	-			-		

a) LD_{50}s are in µg/fly.
b) Resistance Ratio at the level of LD_{50}.
c) LD_{50}s are in ng/fly.
d) Rough estimation because dose-mortality relationship was not log normal.
e) LD_{50} was not determined due to low number of progeny produced by previous generation.
f) Non-selected generation.
g) CS279 substrain collapsed.

Originally it was not the goal of this study to investigate the effect of selection on fecundity. However, by the F_3 generation, a sudden drop appeared in emergence of flies of CS279 sub-strain. Therefore the fecundity of F_3, F_4, F_5, F_6 and F_7 generations was estimated on the basis of unhatched pupae coming from surviving flies after selection. A large decrease in progeny was observed under selection pressure in both strains compared to nonselected parental flies. The number of pupae/female decreased from the value of parental 114 ± 8 to the range of 13-28 in both strains (Table 3). On the other hand a considerable difference was obtained between CARBSEL and CS279 sub-strain in terms of mortality at the pupal stage. While the adult emergence from pupae varied between 88-95 % in each generation of CARBSEL sub-strain, it was reduced from 72 % to 6 % between F3 and F6 generations in the CS279 sub-strain. Presumably, MB-279 acts similarly to the other PMSOs inhibitor, piperonyl butoxide (Staal 1986). The latter caused mortality before adult emergence in

fleshfly (*Neobelliera bullata*) (Darvas *et al.*, 1992) and has insect growth regulator activity in cotton aphids (*Aphis gossypii*) Satoh *et al.*, 1995).

In spite of the fact that the F5 and F6 generations of CS279 sub-strain, which had not been treated with carbofuran+MB-279, produced near normal numbers of pupae (97 and 95 per female, respectively), the mortality at the pupal stage remained high (Table 3). This chronic effect is unexpected and requires further investigation to explain. This effect of MB-279 could play an important role in the management of resistant populations.

Table 3. Influence of the selection on fecundity in CARBSEL and CS279 sub-strains

G	CARBSEL sub-strain					CS279 sub-strain				
	Survivors		Pupae		Emerged	Survivors		Pupae		Emerged
	F [a]	M [b]	Σ	No./female	Flies [c]	F	M	Σ	No./female	Flies
3	625	362	8533	14	7488 (88)	39	54	701	18	507 (72)
4	334	242	7455	22	7087 (95)	7	35	88	13	12 (12)
5	529	244	13915	26	12901 (93)	5 [d]	7 [d]	486	97	93 (19)
6	446	230	12504	28	11477 (92)	39 [d]	54 [d]	3691	95	229 (6)
7	117	89	>5000	nc [e]	nc -	2	0	0	0	0 (0)

[a] Female
[b] Male
[c] Emerged flies expressed as percent of total pupae produced by survivors are in parenthesis
[d] Flies not treated with carbofuran+MB-279
[e] Not counted

The pattern of the synergist spectrum of MB-279 makes a clear distinction among the group of insecticides investigated. As a general rule, in the susceptible WHO/SRS strain, a high degree of synergism was obtained with the aromatic carbamates (SR>10) but smaller ones (SR ≤10) with aliphatic carbamates (Figure 1). Pyrethroids and the organophosphorous propetamphos showed lower values of synergism (SR=2-5). In contrast to this, MB-279 was ineffective in the elevation the insecticide activity of the organochlorine endosulfan and the macrocyclic lacton ivermectin. The CHXSEL and MD-IX strains had very similar synergist spectra with exceptionally high synergist ratios for two closely related carbamates, carbofuran (SR=530 and 273, respectively) and bendiocarb (SR= 1330 and 270, respectively) (Figure 2, 3). Although, these pattern of synergist spectra could suggest that MB-279 and related compounds are selective carbamate synergists, they are comparable with the well-established pyrethroid synergist, piperonyl butoxide in terms of synergent potency in resistant strains used in the present study (Table 4).

CONCLUSION

The new alkynyl synergists seem to be promising chemical tools for overcoming insecticide resistance. Their synergistic activity is similar to piperonyl butoxide on the pyrethroid, permethrin, but much higher on other insecticides. The laboratory selection studies suggest that these synergists have a dual action. Besides the classic synergistic activity manifested by
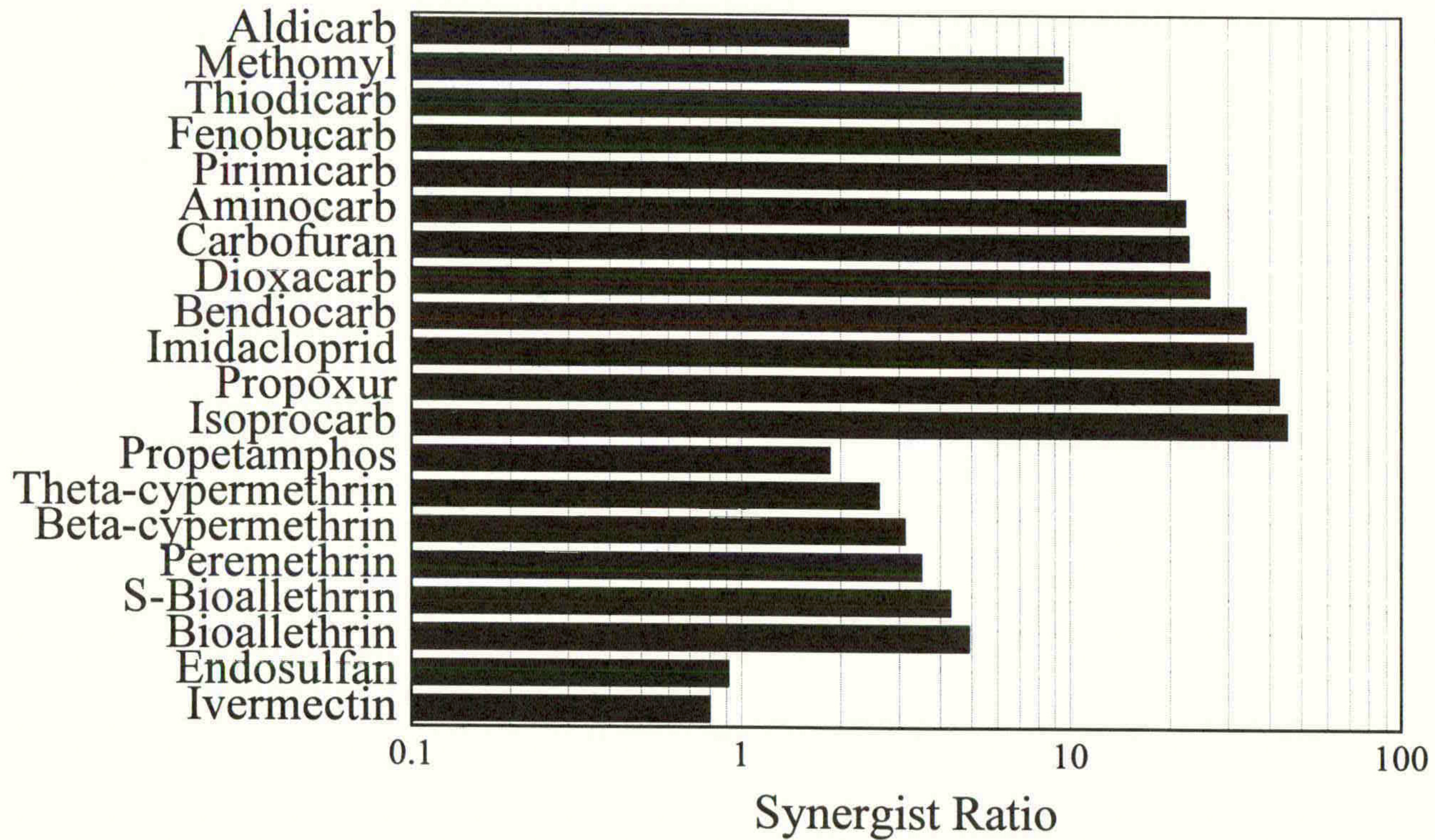
Figure 1.   Synergistic activity of MB-279 on selected insecticides in the susceptible WHO/SRS strain.
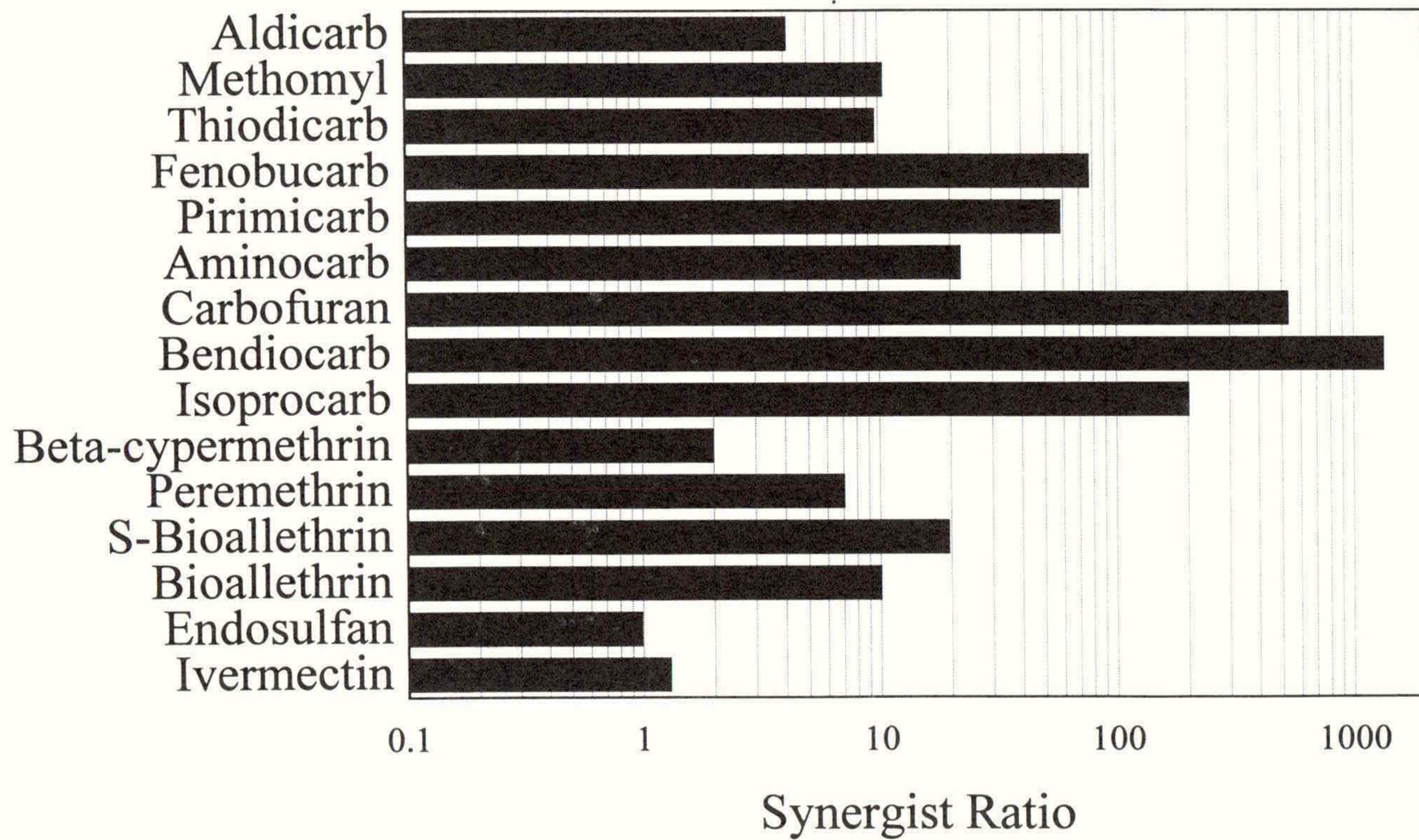
Figure 2.   Synergistic activity of MB-279 on selected insecticides in the pyrethroid resistant CHXSEL strain.
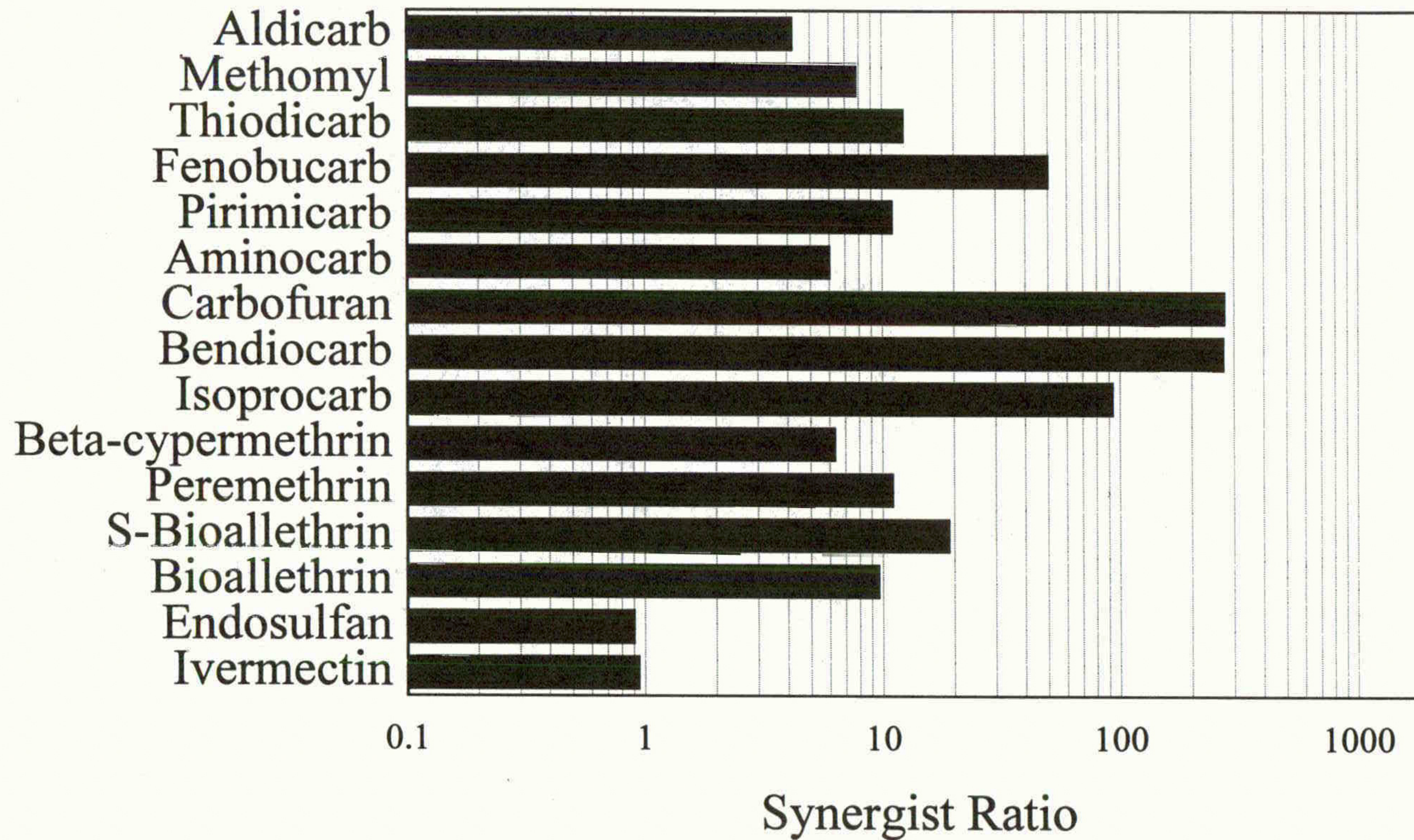
Figure 3.   Synergistic activity of MB-279 on selected insecticides in the multiple resistant MD-IX strain.

increasing toxicity of the insecticide, they have a pronounced chronic effect on insect fecundity. Investigation of these synergists for pest control, particularly for management of resistance populations under field conditions is continuing.

Table 4. Synergistic potency on carbofuran and permethrin in housefly strain having different susceptibilities

Compound	Housefly strains							
	WHO/SRS[a]		CXHXSEL[b]		MD-IX[c]		CARBSEL[d]	
	LD_{50}[e]	SR[f]	RR[g]	SR	RR	SR	RR	SR
Carbofuran	174	-	88	-	147	-	575	-
Carbofuran + PBO[h]	20	9	-	-	23	57	375	13
Carbofuran + MB-603[i]	7	25	-	-	-	-	-	-
Carbofuran + MB-113	7	25	142	15	893	4	25	578
Carbofuran + MB-279	8	22	4	528	12	272	14	905
Carbofuran + verbutin	5	35	5	615	17	308	10	2083
Permethrin	28	-	32	-	1631	-	112	-
Permethrin + PBO	7	4	43	3	910	7	35	13
Permethrin + MB-279	7	4	18	7	593	11	37	12
Permethrin + verbutin	9	3	38	3	661	8	27	13

a) Susceptible WHO strain
b) Beta-cypermethrin selected strain (Pap & Tóth, 1995)
c) Field-collected multiple resistant strain (Pap & Farkas, 1994)
d) Carbofuran-selected strain
e) LD_{50}s are in ng/fly.
f) Synergist Ratio at the level of LD_{50}
g) Resistance Ratio at the level of LD_{50}
h) 1000 ng/fly fixed dose of synergist was applied from each synergists.
i) 1,2,4-trichloro-3-(2-propynyloxy)benzene is another referent synergist (Brown et al., 1996)

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