SESSION 3C

PEST AND DISEASE RESISTANCE TO AGROCHEMICALS

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FUNGICIDE SENSITIVITY IN YELLOW RUST OF WHEAT (*PUCCINIA STRIIFORMIS*)

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ABSTRACT

Isolates of *Puccinia striiformis* collected between 1961 and 1990 were tested for sensitivity to triadimenol and fenpropimorph using seedlings sprayed with low doses of fungicide. Isolates varied in their sensitivity, but there was no evidence that sensitivity had declined over time. There were indications that variation in sensititvity was associated with the geographical origin and specific virulence of isolates. Isolates from the north of the U.K., and those possessing virulence for the wheat cultivar Hornet, tended to be more sensitive than isolates from the south and those lacking virulence for cv. Hornet. There was no relationship between the sensitivity of isolates and whether fungicide had been applied to the crop from which they came.The relative sensitivity of two isolates remained constant for a range of triazole fungicides.

INTRODUCTION

Puccinia striiformis, the causal agent of yellow rust, is a major pathogen of winter wheat in the U.K. Significant outbreaks of the disease occur once every three to four years and are most likely when susceptible varieties dominate the wheat acreage. The risk of disease is usually greatest in the east and north-east regions. Although fungicides of the triazole and morpholine groups have been widely used to control yellow rust, there have been no substantiated reports of loss of disease control in the field. No previous studies of fungicide sensitivity in *P.striiformis* are known, but Boyle *et al.*,(1988) reported variation in sensitivity to triazole fungicides in populations of *P.recondita* (brown rust of wheat) and *P.hordei* (brown rust of barley).

The aims of the investigation reported here were to examine the variation in fungicide sensitivity in *P.striiformis*, determine whether sensitivity has altered over the past twenty years and establish a base-line measure of sensitivity against which to gauge future changes.

Variation in *P.striiformis* for virulence for the genetic resistances of cultivars has been monitored since 1967 by the U.K. Cereal Pathogen Virulence Survey, which maintains an extensive collection of isolates, classified on the basis of virulence and origin. These isolates were the main source of material for fungicide sensitivity tests.

METHODS

Screening of isolates collected 1961-1990

Isolates were screened for sensitivity to triadimenol and fenpropimorph using a seedling test. Each test included a standard isolate for reference. Ten seedlings of the universally susceptible wheat cultivars Sappo or Vuka were grown in 2.5" pots. Seedlings were sprayed with fungicide when the first leaf had expanded (7-8 days after sowing). The dose rates used changed during the testing period, but the following rates were common to all tests:

Triadimenol	15.63 mg AI/l	1/40 field rate
Fenpropimorph	187.50 mg AI/l	1/20 field rate
Nil control		

A field application simulator sprayer, delivering 200 $l.ha^{-1}$ at a pressure of 2 bars was used.

24 hrs after spraying, seedlings were inoculated in a rotary spore inoculator, using fresh uredospores of *P.striiformis* mixed with talc. After incubation for 48 hrs at 7°C and high relative humidity, seedlings were transferred to a controlled environment growth room with 16 hrs light at 18°C and 8 hrs dark at 11°C.

Four to five days after the appearance of yellow rust pustules on untreated seedlings (14-15 days after inoculation), the percentage leaf area covered with pustules was assessed on first leaves. For each isolate, the mean infection on treated seedlings was expressed as a percentage of that on the nil control. The value for the standard isolate was then subracted from that for the test isolate to give an index of infection 'I'. Positive values of 'I' indicated higher infection than the standard isolate i.e lower sensitivity, whilst negative values of 'I' indicated lower infection than the standard i.e greater sensitivity.

Isolates were classified according to:

1. Year of collection
2. Whether or not from a fungicide-treated crop (F+ or F-)
3. Geographical location, north or south of River Tyne (N or S)
4. Virulence or avirulence for cv. Hornet (V or A)

Using these criteria, mean 'I' values for groups of similar isolates were compared using a t-test, to indicate associations between sensitivity to fungicides and other characteristics.

Tests of two isolates with contrasting sensitivity to triadimenol for sensitivity to other triazoles

Two isolates, 90/20 (with low sensitivity to triadimenol) and 83/62 (with near average sensitivity to triadimenol) were tested for sensitivity to five triazoles, at dose rates ranging from 1/40 to 1/5 field rate.

RESULTS

Screening of isolates collected 1961-1990

Results were obtained for 291 isolates tested for sensitivity to triadimenol and 268 isolates tested for sensitivity to fenpropimorph. For triadimenol, 'I' ranged from -40 to +71, (approximately 10%-90% of the infection level on the nil control). For fenpropimorph, 'I' ranged from -54

to +41, (approximately 1%-65% of the infection level on the nil control). There was no significant correlation between sensitivity to triadimenol and sensitivity to fenpropimorph.

Mean infection indices for different groups of isolates, and the significance of the differences between them, are given in Tables 1 to 5.

TABLE 1. Mean infection indices for isolates classified according to year.

Fungicide	Year	No. isolates	mean 'I'	comparison	significance
Triadimenol	pre-1989	102	-3.25	pre '89 v '89	P=0.05
	1989	115	0.082	'89 v '90	P=0.01
	1990	74	-4.51	pre '89 v '90	NS
Fenpropimorph	pre-1989	95	-7.33	pre '89 v '89	NS
	1989	111	-7.89	'89 v '90	NS
	1990	62	-9.45	pre '89 v '90	NS

TABLE 2. Mean infection indices for isolates classified according to fungicide application.

Fungicide	Fungicide application	No. isolates	mean 'I'	significance of difference
Triadimenol	+F	73	-2.70	NS
	-F	215	-2.17	
Fenpropimorph	+F	69	-7.54	NS
	-F	196	-8.33	

TABLE 3. Mean infection indices for isolates classified according to geographical location.

Fungicide	Location	No. isolates	mean 'I'	significance of difference
Triadimenol	N	71	-5.07	P=0.05
	S	205	-1.31	
Fenpropimorph	N	66	-16.25	P=0.001
	S	188	-5.17	

TABLE 4. Mean infection indices for isolates classified according to virulence for cv. Hornet.

Fungicide	Virulence for cv. Hornet	No. isolates	mean 'I'	significance of difference
Triadimenol	V	114	-3.82	P=0.05
	A	167	-0.90	
Fenpropimorph	V	102	-12.05	P=0.01
	A	157	-5.11	

TABLE 5. Mean infection indices for isolates classified according to location and virulence.

Fungicide	Location	Virulence for cv. Hornet	No. isolates	mean 'I'	comparison	significance
Triadimenol	N	V	53	-5.42	N,V v S,V	NS
		A	18*			
	S	V	61	-2.42	S,V v S,A	NS
		A	141	-0.75		
Fenpropimorph	N	V	49	-14.97	N,V v S,V	P=0.05
		A	4*			
	S	V	53	-9.35	S,V v S,A	P=0.001
		A	132	-3.31		

* number of isolates too small for valid comparisons

Tests of two isolates with contrasting sensitivity to triadimenol for sensitivity to other triazoles

Results are given in Table 6.

TABLE 6. Percentage leaf area infected with yellow rust (relative to untreated control) for wheat seedlings treated with five triazole fungicides and inoculated with two isolates of *P.striiformis*

Fungicide	Field rate (mg AI/l)	Dose* rate	Isolate 90/20	Isolate 83/62
Triadimenol	625	1/40	78	54
		1/20	63	44
		1/10	54	23
		1/5	30	8
Cyproconazole	400	1/40	49	7
		1/20	15	2
		1/10	3	0
		1/5	0	0
Flusilazole	1000	1/40	99	99
		1/20	99	88
		1/10	76	49
		1/5	37	22
Propiconazole	625	1/40	73	10
		1/20	37	2
		1/10	13	0
		1/5	1	0
Tebuconazole	1250	1/40	13	4
		1/20	3	0
		1/10	0	0
		1/5	0	0

* fraction of full field rate

Infection levels varied between fungicides, but isolate 90/20 produced consistently more disease than isolate 83/62.

DISCUSSION

Isolates of *Puccinia striiformis* collected between 1961 and 1990 varied widely in their sensitivity to low doses of fungicides, equivalent to 1/40 field rate triadimenol and 1/20 field rate fenpropimorph. As might be expected from the different modes of action of triazole and morpholine fungicides, sensitivity to the two was unrelated. There was no evidence of any consistent trend in sensitivity over this period. The only significant differences were between isolates from the 1989 season and those from both earlier and later years. The 1989 isolates appeared to be less sensitive to triadimenol than isolates collected either before 1989 or during 1990. This may have been associated with unusually high levels of fungicide use during the severe epidemic of yellow rust in 1989.

There was no indication that fungicide application to a crop had

influenced the sensitivity of isolates collected from it. However, the analysis of fungicide effects was limited by the lack of detailed information on products and timing of applications. It is also possible that ingress of inoculum from outside the sprayed crop may have masked the effects of selection taking place within the crop.

The most consistent differences in sensitivity were between isolates of different geographical origin and virulence. The possibility of an association between these two characters was recognised, since virulence for cv. Hornet was first detected in the north in 1988 and became common in this region before establishing further south (Bayles *et al.*,1989, Bayles and Stigwood, 1991)

Isolates from the south tended to be less sensitive to both chemicals than isolates from the north. This observation may be partly accounted for by the more intensive use of fungicides on wheat in the south, but may also be related to geographical differences in pathogen virulence. When comparisons between north and south were restricted to isolates of similar virulence (i.e those virulent on cv. Hornet), the geographical contrast was less pronounced.

Isolates virulent on cv.Hornet were more sensitive than avirulent isolates. When the geographical influence was removed by confining comparisons of virulent and avirulent isolates to those from the south, the differential was maintained for fenpropimorph, but not for triadimenol. The fact that virulent isolates were first selected from northern populations, which were themselves relatively sensitive, may be a partial explanation for their greater sensitivity.

There was evidence that sensitivity to different chemicals in the triazole family is related, implying that insensitivity to the older triazoles would confer insensitivity to newer products.

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REFERENCES

Bayles, R.A.; Stigwood, P.L. (1991). Yellow rust of wheat. 90, 15-20. *United Kingdom Cereal Pathogen Virulence Survey Annual Report for 1990*, 15-20.

Bayles, R.A.; Channell, M.H.; Stigwood, P.L. (1989). Yellow rust of wheat. *United Kingdom Cereal Pathogen Virulence Survey Annual Report for 1988*, 11-15.

Boyle, F.; Gilmour, J.; Lennard, J.H.; Clifford, B.C.; Jones, E.R.L. (1988). Sensitivity of cereal brown rust fungi to triadimefon and propiconazole. *Proceedings of the 1988 Brighton Crop Protection Conference - Pests and Diseases*, **1**, 379-384.

LONG TERM MONITORING RESULTS OF WHEAT POWDERY MILDEW SENSITIVITY TOWARDS FENPROPIMORPH AND STRATEGIES TO AVOID THE DEVELOPMENT OF RESISTANCE

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ABSTRACT

Both triazole and morpholine fungicides are classified as compounds with a low risk of resistance development. On the other hand, wheat powdery mildew clearly belongs to the group of high risk fungi as far as the development of fungicide resistance is concerned.
Therefore, in order to closely follow the sensitivity development of wheat powdery mildew towards fenpropimorph from the very beginning, long term monitoring programmes were initiated, independently from each other, in Switzerland in 1982 by Maag/CIBA-Geigy and in Germany in 1984 by BASF, which coincided with the official launching of this fungicide in the respective countries. A long term field trial with different spray regimes (i.e. combinations and alternations) of fenpropimorph and triadimenol was started in Germany in 1986 in order to obtain data for possible resistance strategies.
The first signs of a slight reduction in the sensitivity of wheat powdery mildew populations to fenpropimorph became apparent in 1989 in both Switzerland and Germany. As far as counter measures are concerned, data from the long term field trial indicate that triazole/morpholine combinations and alternations are equally well suited to avoid a shift in fenpropimorph sensitivity. For practical reasons the use of combinations, either as tank or ready mixtures, is the preferred and widely recommended anti-resistance strategy.

INTRODUCTION

Both triazole and morpholine fungicides, currently the most important groups of fungicides for the control of cereal powdery mildews in Europe, are classified as compounds with a low risk of resistance development. The current situation generally supports this classification, although in the case of triazoles a shift in the sensitivity of cereal powdery mildew populations was recorded in the early 1980s (Fletcher & Wolfe, 1981; Butters *et al.*, 1984) which resulted in the necessity for counter measures by the mid-1980s (Heaney *et al.*, 1988).

Therefore, monitoring programmes for fenpropimorph were initiated with the official launching of this product in Switzerland in 1982 and in Germany in 1984 (Lorenz & Pommer, 1984). Since both programmes have been run independently from each other, the methods and aims are slightly different.

In addition to this, in Germany a specially designed long-term field trial was initiated in 1986, in which in each year and in the same plots the influence of fenpropimorph, alone and in combination or alternation with triazoles, on the sensitivity of powdery mildew populations has been studied. This trial was established in order to obtain data upon which resistance strategies could be based.

MATERIALS and METHODS

Maag/CIBA-Geigy - Switzerland

To follow the development of the sensitivity situation of wheat powdery mildew in Switzerland, bulk samples of powdery mildew were collected at the end of June every other year in the areas of Zürich, Schaffhausen and Solothurn from commercially fenpropimorph-treated fields. These were transferred on the same day to 7-day old wheat plants (cv. Probus) by rubbing the mildew colonies against the leaves of the seedlings in a sterile bench. To avoid cross contamination, the isolates were kept apart by isolating the pots of plants with PVC-tubing. To test sensitivity, plants were sprayed to run-off with a range of fenpropimorph concentrations: 0.3, 1.3, 10, 30, 60 and 100 mg/l AI. After the plants had dried, they were inoculated by passing infected leaves through the seedlings. The inoculated plants were again covered with PVC-tubes and transferred to a glasshouse for about seven days.

A reference isolate was included in each test to check the variability of the results. Assessments were made by estimating the percentage of powdery mildew coverage on the primary leaves per pot and MIC (minimal inhibitory concentration) -values of each isolate were recorded.

BASF-Germany

Bulk samples of powdery mildew were also collected each year at growth stage (GS) 26-30 (Zadoks *et al.*, 1974) from 10 different locations within Germany. At the time of collection, the plants had not been treated. The powdery mildew was immediately transferred onto primary leaf segments of untreated wheat seedlings (cv. Kanzler) inserted into an agar medium, which contained 10 ppm benzimidazole, in Petri dishes.

To test sensitivity, segments of primary leaf of wheat seedlings were used which had been treated to run-off with a concentration range of fenpropimorph: 0.1, 0.5, 1, 5, 10, 50, 100, 250 mg/l AI. The leaf segments, inserted in benzimidazole agar, were inoculated using dry spores and a settling tower. The Petri dishes were then placed in a climate chamber at 18° C and 8000 lux for 14 h/day.

Results were recorded after 8-10 days by counting the number of powdery mildew pustules on the leaf segments. LD98-values were calculated by probit analysis from dose-response curves.

In a long-term field trial with the wheat variety Disponent, which was started in 1986 at a site near the BASF Agricultural Research Station Limburgerhof, the following treatments were included:

I = untreated
II = triadimenol at 125 g/ha
III = fenpropimorph at 750 g/ha in alternation with triadimenol at 125 g/ha
IV = fenpropimorph at 375 g/ha in combination with triadimenol at 125 g/ha
V = fenpropimorph at 750 g/ha

The same plots were used for the same treatments each year. The plot size was 5000 m^2. All plots were sprayed with the onset of powdery mildew attack using standard field-spraying equipment. Applications were repeated in the different plots whenever new powdery mildew infections were recorded. Bulk samples of powdery mildew were collected before the first fungicide application (GS 26-30) and after the last application (GS 68-69). Visual assessments of the infected leaves were recorded as percentage leaf area affected for the entire plot. The trials were harvested using a small plot combine harvester and yields were corrected to 86% dry matter.

RESULTS

The data listed in Table 1 are those obtained from the first sampling at the beginning of the growing season in each year from 10 different regions in Germany. The 1984 values practically represent the base-line data of the powdery mildew populations, since to this date there had not been any large volume/acreage treatment with fenpropimorph. Notable is the relatively wide variation in the reaction of the powdery mildew populations from the different sites. This obviously represents the natural variation within the populations, a factor which was largely maintained until 1988.

TABLE 1. Monitoring data (LD98-values in ppm AI) of wheat powdery mildew and fenpropimorph from different regions in Germany for the years 1984-1988

Location	1984 LD98	1985 LD98	1986 LD98	1987 LD98	1988 LD98
Kiel	-	8	8	2	7
Oldenburg	15	-	-	-	14
Münster	11	-	10	12	4
Hannover	13	15	14	12	3
Köln	8	5	7	4	-
Giessen	-	8	6	3	3
Böhl	8	7	7	5	-
Erlangen	3	5	-	-	2
Stuttgart	-	4	4	-	-
München	7	7	-	2	-

In Table 2, values from all samples from one season in Germany and Switzerland have been combined and arranged into sensitivity classes. There is a remarkable comparability of the sensitivity distributions in both countries, especially when considering the fact that for Germany LD98- and for Switzerland MIC-values are recorded.

TABLE 2. Sensitivity distribution of wheat powdery mildew in Germany and Switzerland (numbers represent percentage of isolates in the respective sensitivity classes)

Year	Germany LD98 (mg/l AI) fenpropimorph							Switzerland MIC (mg/l AI) fenpropimorph						
	No. of isolates	< 1	> 1-3	> 3-10	> 10-30	> 30-60	> 60-100	No. of isolates	< 1	> 1-3	> 3-10	> 10-30	> 30-60	> 60-100
1982	-	-	-	-	-	-	-	84	*	0	30	70	0	0
1983	-	-	-	-	-	-	-	-	*	-	-	-	-	-
1984	14	*	22	57	21	0	0	94	*	1	42	57	0	0
1985	34	*	6	55	39	0	0	-	*	-	-	-	-	-
1986	28	11	82	7	0	0	0	-	*	-	-	-	-	-
1987	35	0	77	17	6	0	0	58	*	0	38	62	0	0
1988	74	3	54	30	14	0	0	-	*	-	-	-	-	-
1989	67	0	21	24	52	3	0	146	*	0	41	46	14	0
1990	137	0	15	32	45	8	0	60	*	0	30	58	11	0
1991	112	0	21	46	30	4	0	73	*	7	44	36	14	0
1992	-							-						

* not tested

TABLE 3. Influence of different spray regimes with morpholines and triazoles on sensitivity and control of wheat powdery and on wheat yield

Treatment	Sampling date: GS 26-30 LD98 (mg/l AI) fenpropi-morph	triadi-menol	GS of treatments	Sampling date: GS 68-69 LD98 (mg/l AI) fenpropi-morph	triadi-menol	% infected leaf area	yield (t/ha)
1986							
I	3	14	-	1	15	32	4,7
II	-	-	47;61	3	30	10	5,9
III	-	-	47;61	3	14	6	6,4
IV	-	-	47;61	3	8	2	6,6
V	-	-	47;61	2	7	2	6,6
1987							
I	8	8	-	2	28	19	5,5
II	2	26	32;47;59	2	45	4	5,4
III	2	3	32;59	2	20	2	5,9
IV	1	3	32;59	2	30	1	5,9
V	2	2	32;59	6	26	1	6,0
1988							
I	4	5	-	6	16	25	4,5
II	8	7	31;37;51;61	4	45	12	6,3
III	2	8	31;51;61	1	52	3	7,8
IV	1	3	31;51;61	3	26	0	7,8
V	1	1	31;51;61	1	4	0	7,9
1989							
I	15	26	-	4	10	15	5,8
II	8	8	30;33;37;65	2	30	11	7,3
III	2	21	30;37;65	2	29	5	8,4
IV	1	7	30;37;65	2	15	0	8,5
V	4	7	30;37;65	12	6	4	8,1
1990							
I	30	39	-	5	27	18	6,4
II	29	58	32;39;59	2	61	5	7,0
III	7	7	32;39;59	6	16	2	7,6
IV	9	31	32;39;59	4	52	2	7,8
V	2	8	32;39;59	18	29	2	7,7
1991							
I	9	93	-	3	15	14	5,7
II	4	28	32;55;65	6	135	5	6,3
III	8	8	36;65	8	30	0	6,8
IV	2	15	32;65	4	29	0	7,0
V	8	31	32;65	33	37	0	6,9

Treatments:

I = untreated
II = triadimenol at 125 g/ha
III = fenpropimorph at 750 g/ha in alternation with triadimenol at 125 g/ha
IV = fenpropimorph at 375 g/ha in combination with triadimenol at 125 g/ha
V = fenpropimorph at 750 g/ha

From 1982 up to 1988 some variability in sensitivity can be seen which is reflected in the numbers of isolates present in the respective classes. It was not until 1989 that a slight but nevertheless real shift towards a decreased sensitivity to fenpropimorph became apparent. This occurred in both countries and was marked by the establishment of a new sensitivity class (30 - 60 ppm) which has remained up to 1991 without any further increase. Thus, apart from this initial shift in 1989 the situation has re-stabilized itself.

Table 3 includes all the assessment data collected from the long-term wheat trial with the variety Disponent over the period 1986-1991. At the beginning of this trial period in spring 1986, a slightly reduced triadimenol sensitivity of the powdery mildew population was recorded at this site. Consequently, there was a distinct reduction in sensitivity in the triadimenol treated plot at the end of the season; this was also reflected in the degree of powdery mildew attack and correlated with the yield.

This effect continued to develop in the following years. From 1987 it became necessary to apply an additional treatment to the triadimenol plot. However, despite the pronounced variation of sensitivity values from year to year, a clear reduction in sensitivity was not observed until 1991.

The sensitivity of the wheat powdery mildew towards fenpropimorph was very high at the beginning of the trial in 1986. The first signs of a slight change in this level of sensitivity were seen in 1987 and in 1989, when this change had become more apparent. This tendency has continued in the following years. A comparison of the yearly values obtained so far in this trial suggests that a not very pronounced, but still clearly distinct shift in the powdery mildew population in its sensitivity towards fenpropimorph has occured, although this is still well within the range of the overall sensitivity distribution, as shown in Table 2. As can be seen from the data relating to % attack and yield, the performance of fenpropimorph remained totally unaffected by this shift.

In contrast to these observations are those recorded from the other two treatments - the alternating spray regime (treatment III) and the combination of fenpropimorph with triadimenol (treatment IV) - where, as yet, there is no indication of a shifting in sensitivity towards fenpropimorph. Both treatments would appear to be equally well suited to avoid this. The same applies with respect to powdery mildew control and triazole sensitivity in these two treatments, although values differ slightly from one year to the other.

DISCUSSION

Whereas in the period from 1982 to 1988 in Germany and in Switzerland there were only insignificant, if any, changes in the sensitivity of wheat powdery mildew populations towards fenpropimorph, the first signs of a sensitivity shift became apparent in 1989. Although this development has re-stabilized itself and the field performance of fenpropimorph, when applied according to label recommendations, has remained unaffected. Nevertheless increased attention must be paid to employ resistance strategies, such as alternating spray regimes or the use of product combinations. This is especially important in view of the fact that the selection pressure exerted by morpholine or triazole treatments alone could be clearly shown in a long-term field trial especially designed to deal with these questions. The trial results obtained so far indicate that both strategies, if handled properly, produce the desired effect. For practical reasons the use of triazole/morpholine combinations, either as tank or ready mixtures, is the preferred and widely recommended anti-resistance strategy.

REFERENCES

Butters, J.; Clark, J.; Hollomon, D.W. (1984) Resistance to inhibitors of sterol biosynthesis in barley powdery mildew. *Mededelingen van de Faculteit Landbouwwetenschappen Rijksuniversiteit Gent,* **49**, 143-151.

Fletcher, J.T.; Wolfe, M.S. (1981) Insensitivity to triadimefon, triadimenol and other fungicides. *Proceeding British Crop Protection Conference, Pests and Diseases,* 633-640.

Heaney, S.P.; Martin, T.J., Smith, J.M. (1988) Practical approaches to managing anti-resistance strategies with DMI fungicides. *Proceedings Brighton Crop Protection Conference, Pests and Diseases,* 1988, **3**, 1097-1106.

Köller, W.; Scheinpflug, H. (1987) Fungal resistance to sterol biosynthesis inhibitors: A new challenge. *Plant Disease,* **71**, 1066-1074.

Lorenz, G.; Pommer, E.H. (1984) Investigations into the sensitivity of wheat powdery mildew population towards fenpropimorph (monitoring programme). *Proceedings British Crop Protection Conference, Pests and Diseases,* **2**, 489-493.

Zadoks, I.E.; Chang, T.T.; Konzak, C.F. (1974) A decimal code for the growth stages of cereals. *Weed Research,* **14**, 415-421.

PRACTICAL ASPECTS OF RESISTANCE TO DMI FUNGICIDES IN BARLEY POWDERY MILDEW *ERYSIPHE GRAMINIS*

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ABSTRACT

From 1976 until 1988, ADAS conducted field experiments to develop strategies for disease control in spring barley. Part of the work included comparison of fungicides from different chemical groups. The work was carried out partly to detect any practical changes in the level of disease control achieved as a result of changes in the sensitivity of mildew populations. The series of experiments clearly demonstrates the decline in activity of the DMI fungicides over a 12-year period. In the mid 1970s the DMI fungicides gave good control of mildew but by the mid-1980s the activity of the DMI's had declined to a level whereby disease control failure was commonly encountered. In direct contrast to the DMI's, the activity of the morpholine fungicide tridemorph was maintained throughout the period of the experiments.

INTRODUCTION

Changes in the sensitivity of fungal plant pathogens to fungicides are commonly recorded following the widespread use of a product. Where the product belongs to a fungicide group which has members which are already widely used, cross resistance is also often claimed. The degree to which populations of fungal pathogens develop insensitivity determines whether or not field resistance has developed. The demonstration of a shift in sensitivity in laboratory tests does not in itself indicate that resistance has developed in practice.

In the mid 1970s ADAS began a series of field experiments to develop strategies for disease control in spring barley. The experiments involved aspects of fungicide timing in response to disease thresholds and fungicide comparison. The comparison of fungicides from different chemical groups enabled the detection of any practical changes in the level of disease control achieved as a result of changes in the sensitivity of mildew populations. Such changes in sensitivity have been investigated by a number of workers (Fletcher and Wolfe, 1981 ; Heaney *et al.*, 1986 ; Wolfe and Minchin, 1976). The results of such experiments were usually expressed as a range of FD_{50} values (i.e. the fungicide concentration which would give a 50% reduction in the level of disease compared with an untreated control).

Although a wide range of FD_{50} values was detected in these experiments, no change in the field performance of the fungicides had been reported. The work of Fletcher and Wolfe demonstrated a wide range of sensitivities of *E. graminis* populations collected throughout the UK. The major conclusion of the work was that "Although there has not been any obvious loss in effectiveness of these (sterol inhibiting) fungicides due to insensitivity, the trends observed give rise to concern".

Fletcher continued his work in 1982 and in his conclusions he stated that "...the survey provides no clear evidence that the insensitivity of mildew populations to sterol inhibiting fungicides is affecting their field performance". Very shortly after this work, failures to control mildew effectively in ADAS field experiments were commonly detected and within 3 years there was a range of DMI fungicides which were no longer giving acceptable levels of mildew control.

METHODS

The main disease problem on spring barley in the UK is powdery mildew and there is frequently a high correlation between disease levels and yield. The relationship between yield and the level of mildew infection in these experiments frequently gives very high correlation coefficients, often in the region of (-)0.95. This relationship allows the disease control performance of a fungicide to be judged by the yield responses achieved. The relative performance of the DMI fungicides throughout this experiment series was measured against that of tridemorph, a morpholine fungicide used commonly in the UK. Although resistance to morpholine fungicides in mildew populations has been discussed by some workers (Walmsley-Woodward *et al.*, 1979; Brown *et al.*, 1991), the changes in sensitivity which were detected were very small and it is still generally agreed that the level of disease control achieved in field situations remains high.

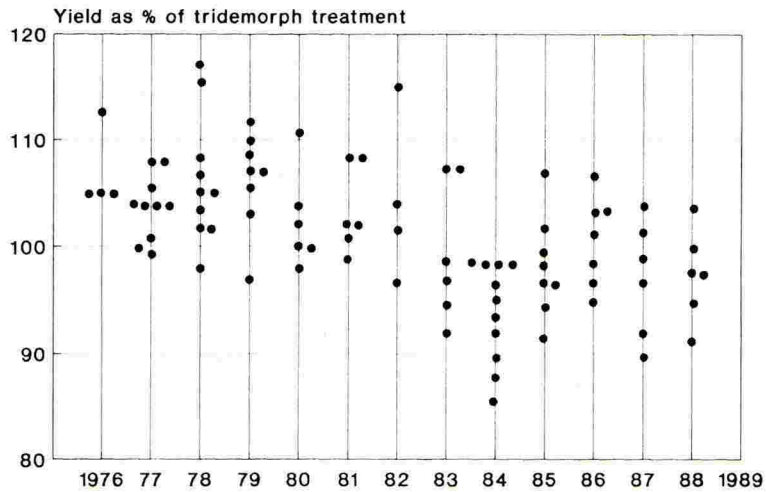
The experiments were conducted in commercial crops of spring barley. A randomised block design with four replicates was used at all sites in all years. Plots were approximately 0.01 ha in size. Fungicides were applied at label recommended rates using knapsack sprayers. Diseases were assessed on 10 tillers per plot and individual leaves were assessed for disease as % leaf area affected using keys from the MAFF Manual of Plant Growth Stage and Disease Assessment Keys. Leaf 1 was taken as the youngest fully expanded leaf (the flag leaf in the mature plant). Plots were combine harvested and yields corrected to 85% dry matter. All data were subjected to analysis of variance and treatment means separated using Duncan's Multiple Range Test.

RESULTS

The performance of triadimefon and triadimenol in controlling barley powdery mildew over the 13-year period of the experiment is represented in Fig.1. This shows the range of yield responses achieved by these products when used to control mildew in crops of spring barley. The yield response is shown as a comparison with that achieved by application of tridemorph in the same experiment at each site. The four experiments in 1976 show clearly that treatment with triadimefon consistently gave higher yield responses than treatment with tridemorph. In most years at most sites, the main disease present was powdery mildew and the yield response at any one site can be largely attributed to the control of mildew.

In 1977, at 10 sites out of 11, application of triadimefon gave yield responses equal to or greater than those achieved by the application of tridemorph. This trend of good performance of triadimefon compared with tridemorph continued until the early 1980's when an increasing number of sites was found where triadimefon gave yield responses below those of tridemorph. By 1984, there had been a total reversal of the relative activities of the products. At all 11 sites, the application of tridemorph gave a larger yield response than where triadimefon had been applied. Over a period of 8 years, the relative activities of the two fungicides against barley powdery mildew had been reversed.

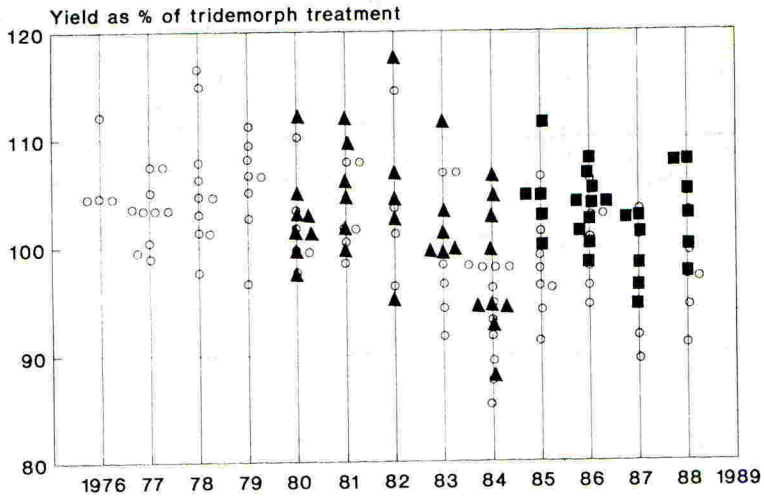
Figure 1. Relative yield response of spring barley to treatment with triadimefon or triadimenol (1976-1988)



Each point on the chart (●) represents the mean yield response to treatment with triadimefon or triadimenol from a fully replicated experiment. (Triadimefon used 1977-83, triadimenol used 1984-88).

Propiconazole, another DMI fungicide with similar activity to triadimefon, was introduced into the experiment series in 1980 and it can be seen from Figure 2 that it follows the trend set by triadimefon in terms of declining activity. Figure 2 shows that although there is an apparent decline in the activity of propiconazole following its introduction into the experiment series its activity was generally better than that of triadimefon. This appears as a lag in the decline of activity when compared with triadimefon. The use of products containing two fungicides with different modes of action did improve the activity against mildew as can be seen in Figure 2 with the introduction of Tilt Turbo (propiconazole + tridemorph) into the experiment series in 1985.

Figure 2. Relative yield response of spring barley to treatment with propiconazole and propiconazole + tridemorph.



Each point represents the mean yield response to treatment with propiconazole (▲) or propiconazole + tridemorph (■). Other points represent those for triadimefon/triadimenol (○) shown in Figure 1.

The level of mildew control and associated yield responses achieved by the application of triadimenol, tridemorph and fenpropimorph in 1979 and 1987 can be seen in Tables 1 and 2. These show the mildew levels and yields at 3 experiment sites in each year where disease levels were present at high levels (>9% leaf area affected). The data illustrate the reversal of relative activities of the DMI and morpholine products over the 8-year period.

DISCUSSION

The series of experiments carried out by ADAS and described here clearly demonstrates the decline in activity of the DMI fungicides (also referred to in earlier papers variously as triazoles and EBI fungicides but belonging to the sterol biosynthesis inhibitor group of fungicides

which act at the point of C_{14} demethylation). The experiments also support the view that there has been no comparable shift in the sensitivity of populations of *E. graminis* to the morpholine fungicide tridemorph over the same period.

The introduction of the 'newer' DMI fungicides such as propiconazole in the early 1980s came at a time when fungicide resistance in spring barley mildew populations had reached a level where failure to control mildew with DMI fungicides was commonplace. The slight improvement in activity following their introduction was therefore shortlived.

Recently published work (Brown *et al.*, 1991) claimed to clearly identify isolates of *E. graminis* collected from Scotland which were 'resistant' to fenpropimorph and fenpropidin. However, the level of resistance was low in relation to field application rates and there was no suggestion of failure of disease control. The same report stated that there was comparatively little variation in the sensitivity of isolates to tridemorph. This work confirmed the value of using tridemorph as a standard in this experiment series against which other fungicide performance could be measured.

The morpholine fungicides, like the DMI's, act by inhibiting sterol biosynthesis but do not act at the C_{14} demethylation point. Members of the morpholine group do not all act in the same way and there is good evidence that tridemorph acts differently from fenpropimorph and fenpropidin, its main activity being the inhibition of sterol $\Delta^8 \rightarrow \Delta^7$ isomerase (Brown *et al.*, 1991). Although the point of inhibition in the pathway is different from that of the DMI's, this alone cannot explain why their activity has remained relatively unaffected in the last decade. Mildew control in cereals now relies almost totally on the morpholine fungicides. We must guard their activity with rational fungicide policies which minimise pressure on the pathogen population and prevent the decline in activity which followed the widespread use of their cousins the DMI's.

TABLE 1. Mildew levels and yield at each of 3 sites showing the effect of fungicide treatment (1979).

	Site 1		Site 2		Site 3	
Treatment	% Mildew*	Yield+	% Mildew*	Yield+	%Mildew*	Yield+
Untreated	10.0 c	4.66 a	28.3 e	4.25 a	9.7 c	6.04 a
triadimefon	0.1 a	5.23 bc	1.1 ab	5.49 d	0.0 a	6.34 a
tridemorph	4.1 ab	4.96 b	13.4 d	5.02 c	0.8 ab	5.97 a
fenpropimorph	0.3 a	5.04 b	5.7 abc	4.69 b	-	-

* % Mildew on Leaf 3, GS 75
\+ Yield in t/ha at 85% DM

(Site 1 : South Milford; Site 2 : Headley Hall; Site 3 : Womersley)

TABLE 2. Mildew levels and yield at each of 3 sites showing the effect of fungicide treatment (1987).

Treatment	Site 1		Site 2		Site 3	
	% Mildew*	Yield+	% Mildew*	Yield+	% Mildew*	Yield+
Untreated	11.9 b	3.08 a	12.4 c	5.17 a	23.1 c	5.64 a
triadimenol	9.0 ab	3.58 b	9.5 c	5.24 ab	20.5 c	5.76 ab
tridemorph	6.6 a	3.86 bc	9.4 c	5.32 abc	14.7 b	6.30 c
fenpropimorph	6.4 a	3.81 bc	8.6 c	5.37 bc	9.8 a	6.34 c

* % Mildew on Leaf 3, GS 75
\+ Yield in t/ha at 85% DM

(Site 1 : East Rigton; Site 2 : High Mowthorpe; Site 3 : Wark)

ACKNOWLEDGEMENTS

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REFERENCES

Brown, J.K.M.; Slater, S.E.; See, K.A. (1991) Sensitivity of *Erysiphe graminis f.sp. hordei* to morpholine and piperidine fungicides. *Crop Protection*, **10**, 445-454.

Fletcher, J.T.; Wolfe, M.S. (1981) Insensitivity of *Erysiphe graminis f.sp. hordei* to triadimefon, triadimenol and other fungicides. *Proceedings British Crop Protection Conference - Pests and Diseases* 1981, *2*, 633-640.

Heaney, S.P.; Hutt, R.T.; Miles, V.G. (1986) Sensitivity to fungicides of barley powdery mildew populations in England and Scotland : Status and implications for fungicide use. *Proceedings British Crop Protection Conference - Pests and Diseases* 1986, **2**, 793-800.

Walmsley-Woodward, D.J.; Laws, F.A.; Whittington, W.J. (1979) The characteristics of isolates of *Erysiphe graminis f. sp. hordei* varying in response to tridemorph and ethirimol. *Annals of Applied Biology*, **92**, 211-219.

Wolfe, M.S.; Minchin, P.N. (1976) Quantitative assessment of variation in the field populations of *Erysiphe graminis f.sp.* hordei using mobile nurseries. *Transactions of the British Mycological Society*, **66**, 332-334.

EFFECTS OF CROP HISTORY ON SENSITIVITY TO PROCHLORAZ OF PSEUDOCERCOSPORELLA HERPOTRICHOIDES ISOLATES FROM CEREALS IN WESTERN EUROPE

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ABSTRACT

In vitro dose-response testing of the sensitivity to prochloraz of populations of Pseudocercosporella herpotrichoides from France, Germany, Denmark and the UK showed no major changes in any of the countries between 1985 and 1991. Isolates from UK and Danish trial plots treated with prochloraz during the 1991 season showed no evidence of selection for lower sensitivity when their in vitro responses to 0.5 mg/l prochloraz were compared with those of isolates from untreated plots. The effects of applications made between 1988 and 1990 on the sensitivity of isolates obtained in 1991 were inconsistent. Although the mean growth of isolates from sites treated 3, 4 and 5 times with prochloraz were higher than some of those from untreated plots this was not always the case, as some of the means from untreated plots were equally high. Further investigation is required before conclusions can be drawn on effects of treatment on sensitivity.

INTRODUCTION

Prochloraz is widely used throughout Europe to control the eyespot disease of cereals, caused by Pseudocercosporella herpotrichoides. Since resistance has developed to carbendazim in most European countries, prochloraz is now the only fungicide which will give commercially acceptable control of all strains within the eyespot population. Prochloraz has been used in cereals for approximately 10 years and, as yet, there is no evidence that field activity has been threatened by development of resistance. However, French workers have recently reported the discovery in Northern France of P. herpotrichoides isolates which they claim to possess laboratory resistance, although there is no proof that these have caused a reduction in the level of control given by prochloraz applications (Leroux and Marchegay, 1991).

Since 1984 the Schering eyespot monitoring programme has examined the sensitivity to prochloraz of isolates of the pathogen from field trials and farmers crops in the UK, France, Germany and Denmark. General monitoring results have been reported previously (Gallimore et al., 1987, Birchmore et al., 1990) and the situation in France has been examined in detail in recent publications (Birchmore et al., 1992 a and b). This current paper reviews the overall levels of sensitivity to prochloraz in the 4 countries, from 1985 to 1991, and also examines in detail the effects of selection pressure from prochloraz applications on the sensitivity of isolates from France, the UK and Denmark.

MATERIALS AND METHODS

Approximately 20 infected stems were removed from each plot within a trial, or 50 stems from a farmers field. These were despatched by air-freight to Chesterford Park Research Station, in Essex, England. P. herpotrichoides cultures were then isolated from eyespot lesions by the method reported by Birchmore (1991).

A two-step, in vitro, testing procedure was used to evaluate the sensitivity of isolates to prochloraz. This consisted of a preliminary step in which the response of each of the isolates to 0.5 mg/l prochloraz incorporated into agar was evaluated, followed by a dose-response test which more accurately defined the responses of randomly selected isolates to prochloraz. The concentrations used were 2.0, 1.0, 0.5, 0.25, 0.10, 0.075, 0.05, 0.0025, 0.01, 0.0075 and 0.005 mg/l prochloraz. Both tests were incubated at 20°C for 14 days and assessed by measurement of colony diameter. The results of both were expressed by growth on treated agar as a percentage of growth on untreated agar. In the case of the dose-response test, these percentages were transformed into logarithmic values and subsequently used in a linear regression analysis to determine the fungicide concentration which inhibited growth by 50% (IG_{50} values).

The results of the dose response test were used for the distributions in Figure 1. However, these results are based on relatively small numbers of isolates, so for the detailed comparisons in Figure 2 and Tables 1, 2 and 3, the initial 0.5 mg/l prochloraz data were used as these are based on larger sample sizes.

RESULTS AND DISCUSSION

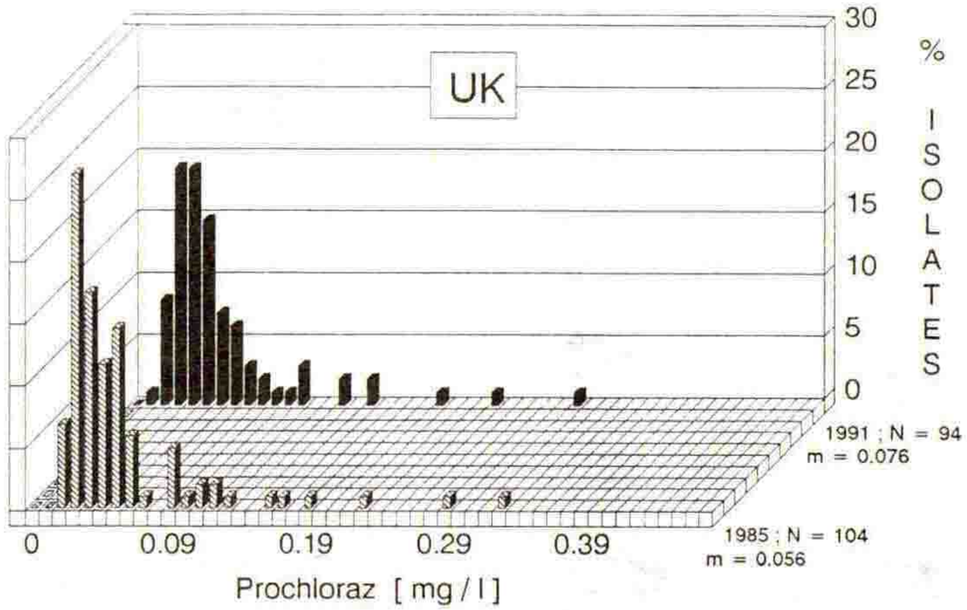
Sensitivity distributions

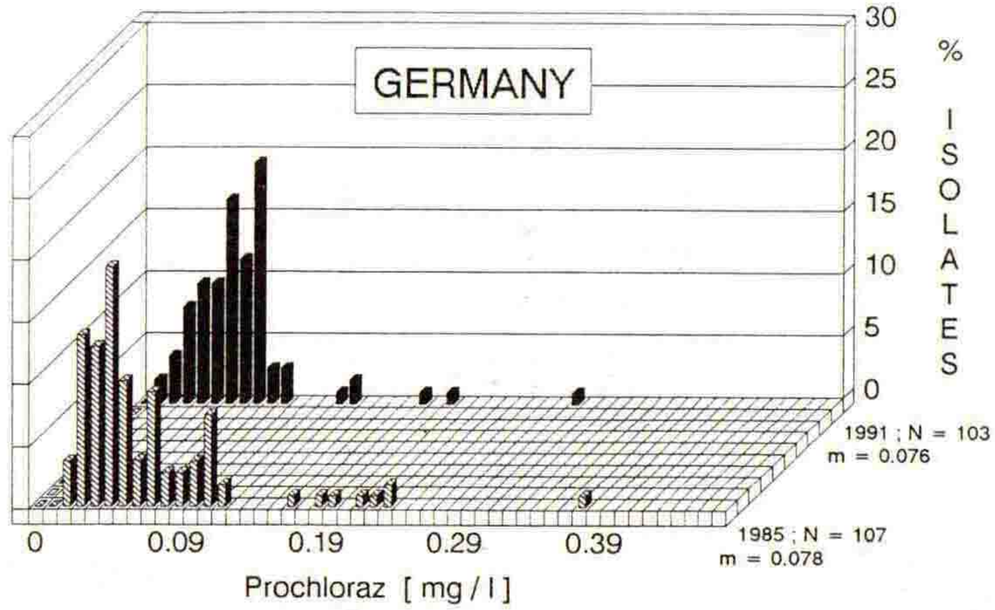
The distribution of prochloraz IG_{50} values obtained from the dose-response testing of cultures from the UK, France, Germany and Denmark are shown in Fig 1. The 1985 and 1991 distributions are shown to illustrate the similarity of the responses, despite the seven-year interval between them. The 1985 Danish distribution showed a higher level of sensitivity than the others, probably due to the small number of isolates tested. The 1991 Danish distribution was very similar to the 1985 distributions from the other countries.

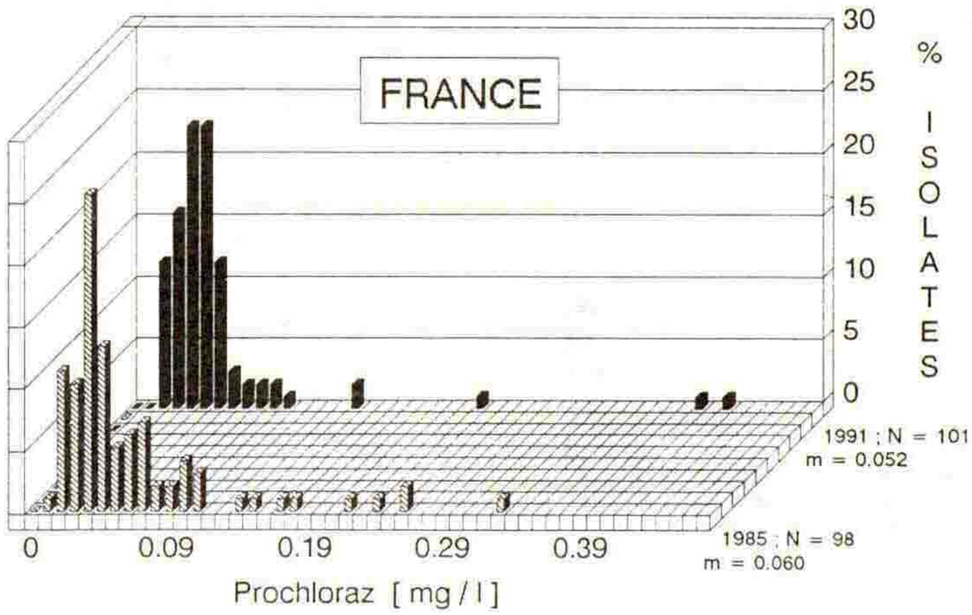
Influence of fungicide applications

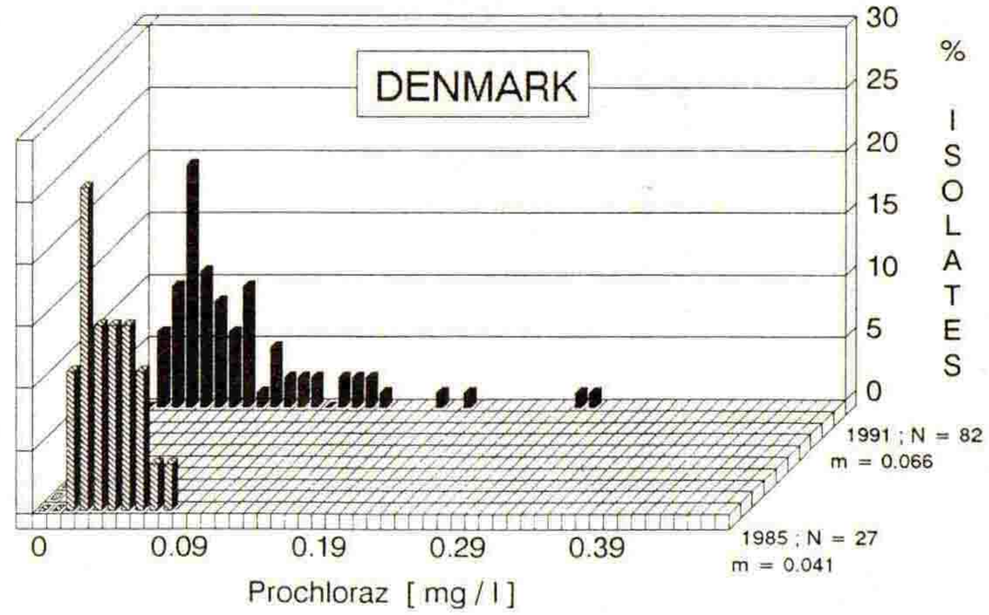
A number of fungicide applications may be made to a cereal crop during the growing season, from seed-treatment to flag-leaf spray. Many of these, while not giving commercially acceptable control of eyespot, may have an effect on the pathogen population. However, a major selection pressure must come from applications of prochloraz, which are intended to control the pathogen. Results of an examination of the effects of prochloraz applications on French eyespot isolates have been published (Birchmore et al., 1992 a and b).

FIGURE 1. Distributions of prochloraz IG_{50} values of isolates from the UK, France, Germany and Denmark; 1985-1991 (N = number of isolates, M = mean IG_{50} value)







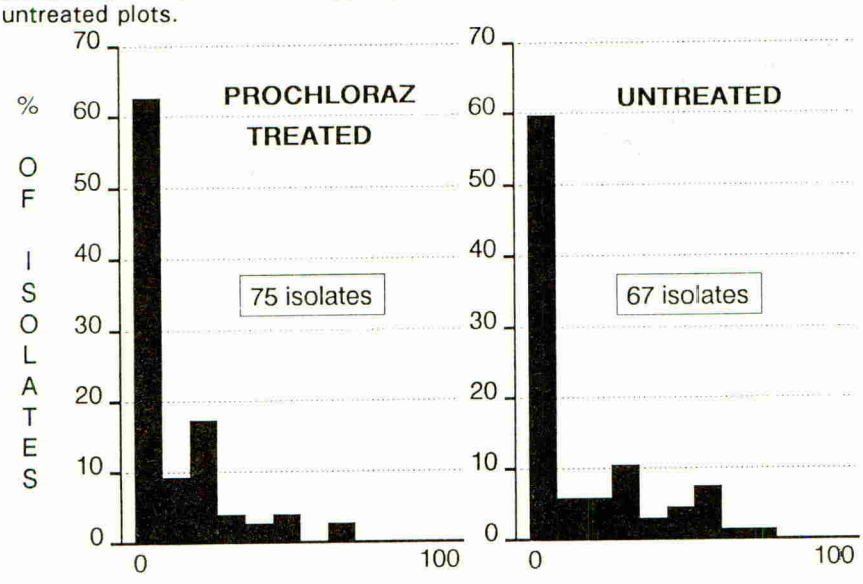


The compound was found to have no detectable effect on the response of isolates to 0.5 mg/l prochloraz in the single dose-rate, in vitro test, either in the season of application or when the effects of applications over a number of years were examined. The conclusions drawn from these data will now be examined further, with reference to UK and Danish trials carried out in 1991.

1991 UK trials

A total of 27 field trials was sampled. The isolates obtained from untreated plots and plots treated with a single application of 450g ai/ha prochloraz were tested in vitro for susceptibility to 0.5 mg/l prochloraz. The distributions of response are very similar (Figure 2) with the least sensitive isolate being obtained from an untreated plot. This conclusion is supported by the mean growth at 0.5 mg/l prochloraz compared with that on untreated agar, which was 13.0% for isolates from untreated plots and 8.7% for those from prochloraz treated plots.

FIGURE 2. Response to 0.5 ppm prochloraz of 1991 UK isolates from treated or untreated plots.



1991 Danish trials

Two types of site were sampled in Denmark during 1991. In the first, fields treated with various numbers of prochloraz applications during 1988 to 1990 were treated and sampled during 1991. In the second, trials were established during the 1991 season, with treated and untreated plots, enabling the effects of treatment during the season and split-rate applications to be evaluated.

Table 1 shows the results of the first set of trials. There was no consistent correlation between the number of prochloraz applications and the mean response to prochloraz.

TABLE 1. Effects of prochloraz applications made between 1988 and 1990 to 10 Danish sites on sensitivity to prochloraz of isolates obtained in 1991.

Site	Number of prochloraz applications in 1988-90 seasons	Number of isolates recovered	Mean colony diameter (mm) at 0.5 mg/l prochloraz as a % of growth on untreated agar
1	0	3	3.0
2	0	8	4.8
3	0	9	38.0
4	0	2	17.8
5	1	2	0
6	1	6	8.7
7	2	5	8.4
8	3	8	42.4
9	4	6	35.9
10	5	3	40.8

The mean colony diameter on sites which had received no prochloraz applications varied from 3.0 to 38.0% of untreated, while those for 3, 4 and 5 applications were 42.4, 35.9 and 40.8% respectively. While this could indicate a trend towards lower sensitivity, or a loss of the most sensitive isolates from the population, the evidence is by no means conclusive as the numbers of isolates recovered were small, the mean values from sites receiving 1 and 2 applications were as low, or lower than those from untreated sites, and there was an overlap between the highest mean from an untreated site and the lowest from a treated site. This lack of correlation agrees with data published previously on French sites (Birchmore et al., 1992).

Tables 2 and 3 show the effects of prochloraz applications during the 1991 season on isolate sensitivity. There was no correlation between the amount of prochloraz applied to plots and the in vitro sensitivity of the isolates obtained.

TABLE 2. Effects of prochloraz applications in 1991 season on sensitivity of P. herpotrichoides isolates taken from 3 Danish trials, each containing 8 treatments.

Treatment	No. of applications made in 1991 season	Total quantity of prochloraz (g AI/ha) applied in season	Mean colony diameter (mm) on 0.5 mg/l prochloraz as % of growth on untreated agar
1	0	0	14.3
2	5	337.5	9.64
3	5	225.0	23.13
4	5	337.5	12.07
5	5	112.5	16.13
6	2	450.0	10.43
7	5	300.0	30.49
8	2	225.0	21.54

TABLE 3. Effects of prochloraz applications in 1991 season on sensitivity of P. herpotrichoides isolates taken from 6 Danish trials, each containing 10 treatments

Treatment	No. of applications made in 1991 season	Total quantity of prochloraz applied in season (g AI/ha)	Mean colony diameter on 0.5 mg/l prochloraz as % of growth on untreated agar
1	0	0	14.70
2	0	0	19.03
3	2	373.5	19.00
4	2	450.0	22.95
5	2	450.0	14.98
6	2	225.0	24.04
7	2	225.0	21.71
8	2	450.0	25.42
9	1	450.0	18.85
10	1	497.0	19.32

In conclusion, the evidence from our monitoring results indicates that the mean reaction to prochloraz of European populations of P. herpotrichoides has remained stable over the years 1985 to 1991. Applications of prochloraz to fields over a number of years may reduce the number of very sensitive isolates in a population, although this effect has not been seen in all locations. However, these applications do not lead to the emergence of a new, resistant strain. Applications of prochloraz during the season of sampling did not have any detectable effect on the eyespot population.

REFERENCES

Birchmore, R.J. (1991), Monitoring populations of Pseudocercosporella herpotrichoides for sensitivity to prochloraz. OEPP Bulletin, 21, 313-315.

Birchmore, R.J.; Russell, P.E.; Buschhaus, H. (1990). Long-term monitoring of Pseudocercosporella herpotrichoides populations for sensitivity to prochloraz and carbendazim. Proceedings of the 1990 Brighton Crop Protection Conference - Pests and Diseases, 1153-1158.

Birchmore, R.J.; Russell, P.E.; de Saint-Blanquat, A.; Salembier, E.; Tromas.J. (1992a). Le prochloraze, résultats des travaux sur Piétin-Verse (1985-91). Phytoma 437, 33-36.

Birchmore, R.J.; Russell, P.E.; Ashman, P.I.; Stanley, S.; Buschhaus, H. (1992b). 44th International Symposium on Crop Protection, Gent (in press).

Gallimore, K.; Knights, I.K.; Barnes, G. (1987) Sensitivity of Pseudocercosporella herpotrichoides to the fungicide prochloraz. Plant Pathology 36, 290-296.

Leroux, P.; Marchegay, P. (1991). Caractérisation des souches de Pseudocercosporella herpotrichoides, agent de piétin-verse des céréales, résistantes au prochloraze, isolées en France sur blé tendre d'hiver. Agronomie 11, 767-776.

RESISTANCE OF THE EYESPOT FUNGUS, *PSEUDOCERCOPORELLA HERPOTRICHOIDES*, TO DMI FUNGICIDES

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ABSTRACT

Populations of *Pseudocercosporella herpotrichoides* were monitored for sensitivity to triazoles and to the imidazole prochloraz in 1990 and 1991. The pathogen was isolated from cereal stems obtained in fields in different regions in France. A stable proportion of fast growing strains was resistant to triazoles. Few isolates were resistant to prochloraz in both the fast and the slow growing type. In greenhouse experiments, plants were inoculated with strains resistant to triazoles and prochloraz and then treated with hepoxiconazole, cyproconazole and prochloraz. For the triazoles, the efficacy declined markedly, and the activity of prochloraz was poorer against prochloraz resistant strains. The isolates remain resistant after inoculation on plants. In practice, if the frequency of resistance is increasing, chemical control of eyespot may become unreliable.

INTRODUCTION

Since MBC generating fungicides have become ineffective for eyespot control because of the development of resistance in the pathogen *Pseudocercosporella herpotrichoides*, prochloraz and flusilazole are the most commonly used fungicides for controlling this pathogen.

The pathogen population can be divided according to the growth speed into two types : the fast growing (N) and the slow growing (L) strains. An important variability is observed between the two types : pathogenicity, cultural and conidial characteristics, fungicide sensitivity. L strains are less susceptible than N type to triazoles, (Leroux & Gredt, 1985 ; Cavelier, 1988). However even if most of the N isolates are susceptible to triazoles (so-called Na isolates) some of them (so-called Nb) are resistant to this fungicide group (Leroux & Gredt, 1988 ; Cavelier *et al.*, 1990). Prochloraz has been found to be equally effective against both types, L and N (Leroux & Gredt, 1985 ; Cavelier *et al.*, 1987). However several prochloraz resistant strains were isolated for the first time in France during the year 1990 on winter wheat (Leroux & Marchegay, 1991).

This paper presents the results of characterizing eyespot strains according to the sensitivity of the fungus to triazoles and prochloraz in 1990 and 1991 and describes glasshouse experiments which compare the sensitivity of Na, Nb, L isolates to triazoles and prochloraz.

MATERIALS AND METHODS

1 – Characterization of *P. herpotrichoides* populations

Population of *P. herpotrichoides* were characterized from about 200 treated or untreated plot per year in different regions in France. 50 stems with eyespot lesions were collected per plot. *P. herpotrichoides* isolated from eyespot lesions was identified as L type or N type by colony diameter and colony morphology on potato dextrose agar (PDA Bio–Mérieux) after 14 days growth at 20° C : L type grew at about half the rate of N type isolates and producted colonies with feathery margins. To test the sensitivity of isolates to fungicides, three replicate colonies of each isolate were transferred to three different Petri dishes amended with triadimenol at 30 µg/ml or prochloraz at 0.5 µg/ml or 2 µg/ml. Formulated compounds were used. The cultures were incubated at 20° C for 2 weeks and colony diameters were measured.

2 – Greenhouse experiments

The strains selected for this study were isolated from winter wheat in 1988, 1989, 1990, 1991 in different regions in France. Five isolates were triazole–sensitive N type (Na), five were triazole–resistant N type (Nb), five were prochloraz–sensitive L types (Ls) and five were prochloraz–resistant L type (Lp) isolated by Leroux in the North of France (Leroux & Marchegay, 1992).

Experiments were in a glasshouse heated only to give frost protection. Pots were arranged in a randomised design with five blocks and five plants per pot. Plants, cv. Camp–Rémy, were inoculated at the three leaf growth stage by a suspension of finely chopped mycelium. Treatments were applied four weeks after inoculation. The fungicides tested were two triazoles : cyproconazole at the rate of 100 g AI/ha, hepoxiconazole at the rate of 180 g AI/ha and one imidazole : prochloraz at the rate of 600 g AI/ha.

The severity of eyespot was assessed at GS 60 (Zadoks *et al.*, 1974) by the method of Cavelier and Le Page (1985). Plants were distributed on a 0–2 scale : 0–healthy plants, 1–sheaths with lesions, 2–stems with stroma, a mean score was calculated. On stem, when eyespot lesions were present, the mean proportion of stem cross–section infected was calculated.

RESULTS

1 – Distribution and frequency of Nb strains in France

The Nb type was isolated in all regions of France, with the exception of the North because in this region L type is prevalent. These results were similar to those obtained in 1988–1989 (Cavelier *et al.*, 1990).

The proportion of the Nb type in the N–population seemed to be stable, suggesting the possibility of a rather slow selection for less sensitive strains to triazoles. However, variable

results were observed according to the season, (from 0 to 38 %) and to the region (from 0 to 100 %). In some plots where N type was prevalent, more than half N strains were Nb strains (Table 1).

TABLE 1. Frequency (%) of Nb strains in 1990 and 1991

Departments		Nb strains %		Nb strains % / N strains isolated	
		1990	1991	1990	1991
02	Aisne	22	–	–	–
03	Allier	0	–	–	–
08	Ardennes	100	–	–	–
10	Aube	15	0	–	–
14	Calvados	–	6	–	17
17	Charentes maritime	68	32	64	25
21	Côte d'Or	0	38	–	72
22	Côtes d'Armor	0	6	–	67
28	Eure et Loir	8	20	27	39
29	Finistère	0	–	–	–
31	Garonne (haute)	0	12	–	24
35	Ille et Vilaine	5	0	36	–
37	Indre et Loire	34	16	–	37
41	Loir et Cher	0	–	–	
45	Loiret	0	32	–	50
49	Maine et Loire	0	–	–	–
51	Marne	63	25	68	39
53	Mayenne	–	21	–	29
56	Morbihan	14	–	–	–
60	Oise	2	–	–	–
62	Pas de Calais	0	0	–	–
76	Seine maritime	0	0	–	–
77	Seine et Marne	–	16	–	45
78	Yvelines	19	17	19	39
80	Somme	–	0	–	–
86	Vienne	10	–	–	–
91	Essonne	0	–	–	–

2 – Frequency of isolates resistant to prochloraz

In 1990 a very low percentage of isolates were found which grew on agar amended with 0.5 or 2 μg/ml of prochloraz.

In 1991, the percentage of isolates growing on prochloraz was consistently higher, especially on 0.5 μg/ml and with both types N and L (table 2). These results differ from the

observations of Leroux and Marchegay (1991) ; the test used was not the same but, the number of isolates tested was higher and came from different regions in France, not only from the North.

TABLE 2. Percentage of isolates growing on 0.5 μg/ml or 2 μg/ml of prochloraz

Strain type		1990	1991
L	0.5	0	4.8
	2	0.1	1.5
N	0.5	2.4	7.1
	2	0	2.2
Number of plots tested		109	182

3 – Effects of DMI fungicides on different types of *P. herpotrichoides* in glasshouse experiments (Table 3)

TABLE 3. Effects of fungicides on different types of *P. herpotrichoides*

Strain type		Mean disease score		mean proportion of stem cross–section infected	
Na	untreated	1.98	a*	53.5	a*
	Hepoxiconazole	0.94	b	23.7	b
	cyproconazole	0.90	b	16.7	b
	Prochloraz	0.32	c	5.9	c
Nb	untreated	1.80	a	55.2	a
	Hepoxiconazole	1.56	b	44.6	b
	cyproconazole	1.51	b	39.4	b
	Prochloraz	0.39	c	8.2	c
Ls	untreated	1.45	a	18.1	a
	Hepoxiconazole	1.39	a	15.5	a
	cyproconazole	1.38	a	23	a
	Prochloraz	0.23	b	2.1	b
Lp	untreated	1.70	a	34.3	a
	Hepoxiconazole	1.55	a	27.1	a
	cyproconazole	1.10	b	17.5	b
	Prochloraz	1.08	b	12.6	b

* significant (p = 0.05)

Hepoxiconazole and cyproconazole decreased eyespot incidence in both samples Na and Nb but eyespot severity remained high in Nb plots (Table 3). Prochloraz was the most effective in both cases.

Triazoles failed to control eyespot in plots inoculated with L type, prochloraz was effective against sensitive L type but was less effective in plots inoculated with resistant L type.

No significant difference was observed between hepoxiconazole and cyproconazole.

The mean IG 50 value of L isolates resistant to prochloraz was about 0.3 μg/ml on PDA (0.04 μg/ml for the sensitive strains) ; with a resistance level of 7.5 the efficacy of prochloraz decreased from 84 % to 36 % (calculated on disease score).

The pathogenicity of resistant isolates was at least the same as that of sensitive isolates (Table 4)

TABLE 4. Pathogenicity of DMI – sensitive or resistant isolates of *P. herpotrichoides*

Isolate type	Mean disease score		Mean proportion of stem cross-section infected	
Na	1.98	a *	53.5	a *
Nb	1.80	ab	55.2	a
Lp	1.70	b	34.4	b
Ls	1.38	c	23.0	c

*Significant (p = 0.05)

DISCUSSION

The mean frequency of Nb strains seemed to be stable, but the variability was high between the seasons and between the regions. After artificial inoculation, the efficacy of cyproconazole and hepoxiconazole fell severely. These results confirms the results of previous experiments (Cavelier *et al.*, 1990) in wich fluzilazole was used instead of hepoxiconazole and the sensitivity of Nb and L isolates was nearly the same. This suggests that for a single isolate the resistance is stable and that with a high proportion of Nb strains in a field the efficacy of triazoles is not sufficient to control eyespot. The frequency of isolates resistant to prochloraz was rather low, but also in this case, after artificial inoculation the resistance of resistant isolates persisted, the efficacy of prochloraz fell. Even if the level of resistant isolates is not very high and even if a fluctuation in the distribution of IG 50 values was previously observed (Birchmore *et al.*, 1990), the risk of development of resistant strains to prochloraz in the eyespot population exists. However, in the plots tested the percentage of resistant isolates was too low to explain a possible decrease of the efficacy of prochloraz.

It is now well known that eyespot population is not stable. We do not know the mechanism of selection of fast growing strains resistant to triazoles (Nb type) and a crucial threshold is perhaps reached with the use of prochloraz. If the frequency of resistance is increasing, chemical control of eyespot may become unreliable.

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REFERENCES

Birchmore, R.J. ; Russell, P.E. ; Buschhaus, H. (1990) Long-term monitoring of *Pseudocercosporella herpotrichoides* populations for sensitivity to prochloraz and carbendazim. 1990 British Crop Protection Conference - Pests and Diseases, **3**, 1153-1158.

Cavelier, N. (1988) Variability of the sensitivity of different isolates of *Pseudocercosporella herpotrichoides* to sterol biosynthesis inhibitors fungicides. Conference of the Cereal Section of EUCARPIA, Wageningen, 1988.02.24-26 in : Cereal breeding related to integrated cereal production, Pudoc Wageningen, 140-144.

Cavelier, N. ; Le Page, D. (1985) Caractéristiques de souches de *Pseudocercosporella herpotrichoides* (Fron) Deighton (agent du piétin-verse des céréales) résistances aux fongicides benzimidazoles et thiophanates : pouvoir pathogène, capacité de développement. ANPP, 1ères journées d'études sur les maladies des plantes, **1**, 49-56.

Cavelier, N. ; Lucas, E. ; Prunier, M. (1990) Sensibilité de différents isolats de *Pseudocercosporella herpotrichoides* à des fongicides inhibiteurs de la biosynthèse de l'ergostérol. Mededelingen van de Faculteit Landbouwwetenschappen Rijksuniversiteit Gent, **55**, 989-995.

Cavelier, N. ; Rousseau, M. ; Le Page, D. (1987) Variabilité de *Pseudocercosporella herpotrichoides*, agent du piétin-verse des céréales : comportement *in vivo* de deux types d'isolats et d'une population en mélange. Zeitschrift für Pflanzenkrankheten und Pflanzenschutz, **94**, 590-599.

Leroux, P. ; Gredt, M. (1985) Caractéristiques des souches de *Pseudocercosporella herpotrichoides*, agent du piétin-verse des céréales, résistantes à des inhibiteurs de la biosynthèse des stérols. Comptes-rendus de l'Académie des Sciences - Paris, **301**, 785-788.

Leroux, P. ; Gredt, M. (1988) Caractérisation des souches de *Pseudocercosporella herpotrichoides*, agent du piétin-verse des céréales résistantes à des inhibiteurs antimitotiques et à des inhibiteurs de la biosynthèse des stérols. Agronomie, **8**, 719-729.

Leroux, P. ; Marchegay, P (1991) Caractérisation des souches de *Pseudocercosporella herpotrichoides*, agent du piétin-verse des céréales au prochloraze, isolées en France sur blé tendre d'hiver. Agronomie, **11**, 767-776.

Leroux, P. ; Marchegay, P. (1992) Variabilité chez l'agent du piétin-verse des céréales : implications pratiques. Phytoma - La Défense des Végétaux, **437**, 25-28.

Zadoks, J.C. ; Chang, T.T. ; Konzak, C.F. (1974) A decimal code for the growth stages of cereals. Weed research, **14** 415-421.

SENSITIVITY OF APPLE POWDERY MILDEW (*PODOSPHAERA LEUCOTRICHA*) TO TRIADIMEFON

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ABSTRACT

Sensitivity tests with triadimefon against apple powdery mildew *Podosphaera leucotricha* have been conducted throughout the years 1991 and 1992 on isolates from England, Belgium, The Netherlands and Germany. The data demonstrate that failures in disease control in practice are not due to field resistance of the pathogen to the fungicide. The performance of triadimefon in a long-lasting trial in South Tyrol (1973 to 1991) was continuously good despite intensive applications.

INTRODUCTION

Poor spray scheduling or wrong fungicide dosages often have been the cause for reports of decreasing performance of azole fungicides in apple powdery mildew. Nevertheless, it has been suspected that the selection of resistant mildew strains has negatively influenced azole performance. This has been investigated throughout the years 1991 and 1992. Isolates from England, Belgium, The Netherlands and Germany were tested for sensitivity to triadimefon.

METHODS

Twig-tips or leaves infected with powdery mildew were taken from commercial orchards. These samples were either examined directly in a whole plant test or first transferred to plants for propagation of the inoculum and then tested after one step of subculture where possible.

Young apple seedlings, variety 'Morgenduft' were used. Two weeks after transplantation the little plants had four well developed leaves and were suitable for the test.

The test plants were sprayed with two discriminating dosages of triadimefon (as (R)Bayleton 25 WP) : 100 and 10 mg/l, and sometimes in addition with 50 or 30 mg/l respectively. Control plants were sprayed with water + blank formulation. The plants were inoculated after the spray deposit had dried, using a spore-suspension of each mildew sample prepared in water. In each test a standard-isolate was included. This isolate had been taken from an orchard near Monheim and is sensitive to azole fungicides.

The inoculated plants were covered with cellophane bags which were not opened during the whole test run. The plants were incubated in a greenhouse or growth chamber at 22 °C.

The evaluations were carried out about 10 to 14 days after inoculation. The efficacy was assessed either on whole plants or younger and older leaves separately.

RESULTS

In Tables 1 to 3 representative results are presented which were obtained with samples from The Netherlands and England. In 1990 the fungicidal performance was not satisfactory in the English orchard 'Gore Farm' but was satisfactory in the neighbouring 'Bridge Orchard'.

First of all it is noticeable that many mildew samples grew badly if at all. This is particularly marked for the samples from the English orchards (Tables 2,3). The mildew inoculum of twig-tips taken from 'Bridge Orchard' did not produce any infections in the test. This indicates that although the mildew could be seen macroscopically it had largely been killed by the fungicide treatments in the orchard.

The fungicide concentrations in the test have been chosen in such a way that the standard mildew isolate is controlled completely at 100, 50 and 30 mg triaidmefon/l and the efficacy decreases at 10 mg/l under the standard test conditions. Comparing the Abbott values at 10 mg triadimefon/l, within a certain variation the control of the isolates from the orchards was very good to quite satisfactory independent of the method of assessment. The range of efficacies was greater with English isolates than with the Dutch ones. 100 % control was achieved at 50 mg/l (Table 1) and very good efficacy at 30 mg/l.
A certain variation in sensitivity of the different mildew isolates is normal. Therefore the results do not indicate any resistance in practice and give no cause for concern.

The long-lasting efficacy of different triadimefon formulations compared to sulphur is shown in Table 4. This trial has been running for over twenty years now and a large number of triadimefon sprays have been applied annually. The efficacy of the azole is still excellent. In this orchard no mildew isolates have accumulated which are resistant to azoles despite this high selection pressure.

DISCUSSION

Whenever a problem comes up in practice that cannot be explained for the moment, the question is asked whether a pathogen has become resistant to azole fungicides. Under certain conditions azoles are able to select resistant pathogen strains, as has been experienced with powdery mildew in cereals. In trying to recognize problems early enough and to avoid them, it makes sense to follow up the 'resistance question' for apple powdery mildew as well.

In this paper some results of the resistance research are presented as an example. Further tests with isolates from Belgium and Germany confirm the conclusions.
The available data demonstrate that the reasons for bad perfomance in some orchards are not due to field resistance of the pathogen to triadimefon.

It is necessary that growers reconsider critically any changes in application of plant protection compounds, especially reduced rates of fungicides, reduced water quantities, and the use of ULV sprays.

TABLE 1. **Apple Powdery Mildew - The Netherlands**

Trial in Beesd 1991
Variety: Elstar
Sampling 27.6.91, twig-tips, isolates tested directly

test on whole plants,variety 'Morgenduft' with triadimefon

treatment isolate	Sensitivity test older leaves % attack control	older leaves % efficacy mg triadimefon/l 100	older leaves % efficacy mg triadimefon/l 10	younger leaves % attack control	younger leaves % efficacy mg triadimefon/l 100	younger leaves % efficacy mg triadimefon/l 10
Standard	21	100	82	66	100	90
untreated						
a	67	100	94	81	100	69
b	75	100	92	91	100	55
c	71	100	93	79	100	55
d	82	100	95	92	100	60
Bayleton						
a	55	100	93	66	100	72
b	62	100	84	70	100	78
c	73	100	79	78	100	57
margin	66	100	80	72	100	81

treatments in the orchard: 7x triadimefon (as Bayleton 5WG, 0,05%)
10.4./19.4./2.5./13.5./23.5./5.6./17.6.91(Pyrifenox before bloom)

Plot	number of secondary infect. 125 leaves/repetition a	b	c	d	sum
untreated	64	35	64	39	202
Bayleton	0	2	3	2	7

assessments in the orchard: 3.7.91

Trial in Varik 1992
Variety: Elstar
Sampling 20.5.92, twig-tips
isolates tested directly

test on whole plants, variety 'Morgenduft' with triadimefon

Treatment isolate	Sensitivity test assessment on whole plant % attack control	% efficacy mg triadimefon/l 100	50	10
Standard	52	100	100	88
untreated *				
a	52	100	100	90
b	77	100	100	90
c	70	100	100	85
d	55	100	100	95
Bayfidan *				
a	27	100	100	90
b	15	100	100	95
c	67	100	100	96
d	12	100	100	78

treatments in the orchard:
3x triadimenol (as Bayfidan 50 EW, 0,03%)
29.4./6.5./14.5.92
* = + Captan against scab

27.5.92: assessment of secondary infections in the orchard:97,5% efficacy of triadimenol

TABLE 2. Apple Powdery Mildew 1991 - England

Gore Farm and Bridge Orchard, Kent

Variety: Cox Orange (both farms)
Sampling: 24.4.91, blossom trusses
mildew isolates subcultured once before tests

2 tests on plants, variety 'Morgenduft' with triadimefon

Farm Treatment Isolate	Sensitivity test % attack control	% efficacy (Abbott) mg triadimefon/l 100	 10
Standard	53	100	74
Gore Farm			
untreated			
1	24	99	38
2	13	100	54
3	29	100	52
4	18	97	56
5	20	100	20
6	13	100	50
7	<10	--	--
8	22	100	59
9	12	99	77
10	15	99	30
treated			
1	<10	--	--
2	<10	--	--
3	<10	--	--
4	15	100	62
5	14	99	69
6	19	100	52
7	10	100	74
8	10	99	95
9	<10	--	--
10	15	97	68
Bridge Orch.			
1	27	100	62
2	<10	--	--
3	18	100	23
4	10	99	57
5	18	100	32

treatments:

Gore Farm 1991:
2 winter sprays
penconazole, sulphur
2 spring sprays
myclobutanil
(ULV-sprays, reduced application rates)

history:
triadimefon
penconazole

Bridge Orchard:
history:
triadimefon
penconazole

TABLE 3. Apple Powdery Mildew 1992 - England

Gore Farm and Bridge Orchard, Kent
Varieties: Gore Farm 'Cox Orange' and 'Golden Delicious'
Bridge Orchard 'Cox Orange'

Sampling: 8.6.92, tips of young shoots from treated trees
mildew isolates tested directly without subculture

2 tests on plants, variety 'Morgenduft' with triadimefon

Farm Variety Isolate	Sensitivity test % attack control	% efficacy (Abbott) mg triadimefon/l 100	30	10
Standard	52	100	96	90
Gore Farm				
Golden				
1	--	no mildew infections		
2	<10	--	--	--
3	<10	--	--	--
4	--	no mildew infections		
5	<10	--	--	--
6	<10	--	--	--
7	14	98	98	83
8	<10	--	--	--
Cox				
1	42	99	87	33
2	47	100	95	44
3	77	100	89	48
4	65	100	90	43
5	57	99	83	7
6	27	99	91	69
7	<10	--	--	--
8	<10	--	--	--
9	47	100	86	51
10	17	100	77	71
11	17	100	71	70
12	13	100	100	38
Bridge Orch.				
1	<10	--	--	--
2	--	no mildew infections		
3	--	"		
4	--	"		
5	--	"		
6	--	"		
7	--	"		
8	--	"		
9	--	"		
10	--	"		
11	--	"		

treatments:

Gore Farm 1992:
7x penconazole + nitrothal-isopropyl & metiram
(ULV-sprays with 55 l/ha)

Bridge Orchard 1992:
5x penconazole & dithianon
1x benomyl
1x nitrothal-isopropyl & metiram
1x myclobutanil
(ULV-sprays with 56 l/ha)

TABLE 4. Long-term trial with triadimefon

***Podosphaera leucotricha* - leaf symptoms**

Variety: Jonathan
Site : South Tyrol / Leifers

Year	Number of applications	Efficacy (% Abbott) triadimefon * % a.i. 0,0025	0,0031	0,0062	sulphur 0,18%a.i	Control % attack
1973	14		65	61	59	64
1974	12		83	92	70	64
1975	11		91	95	41	73
1976	10	75	74	88	69	65
1977	12	94	97	97	85	74
1978	12	96	97	98	92	76
1979	15	93	98	98	91	61
1980	15	97	100	100	97	64
1981	12	96	99	100	92	34
1982	11	99	99	100	92	75
1983	11	97	98	100	78	69
1984	10	98	99	100	89	75
1985	12	96	99	99	91	61
1986	9	65	78	92	53	55
1987	12	96	98	99	75	75
1988	11	79	91	95	42	57
1989	12	93	96	98	83	88
1990	12	85	92	96	75	83
1991	12	92	97	98	82	87

* = different formulations
partly in mixture with captan

PHENYLAMIDE RESISTANCE IN *PHYTOPHTHORA INFESTANS* IN NORTHERN IRELAND - A CHANGING SITUATION

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ABSTRACT

Since 1981, the incidence of phenylamide-resistant *Phytophthora infestans* has been surveyed annually using isolates derived from samples of potato blight collected from seed crops throughout the potato-growing areas of Northern Ireland. The percentage of isolates containing resistant strains was below 50% up to 1987 when it increased to over 80%. However, for the last three successive seasons, the proportion has declined from a peak of 90% in 1988 to 42% in 1991. This trend appears to be related to a decline in the usage of phenylamide fungicides and also to weather in July-August not particularly favourable to blight during 1989-1991. The distribution of phenylamide-resistant strains within crops was investigated in 1990 using single lesion isolates. Each of the six crops sampled was found to contain both resistant and sensitive strains, but only one yielded less than 50% resistant isolates.

INTRODUCTION

In Northern Ireland, where a formulation containing the phenylamide fungicide metalaxyl plus mancozeb, introduced in 1978, was widely used against potato blight (caused by *Phytophthora infestans*), phenylamide-resistant strains were first detected in tubers from the 1980 crop (Cooke, 1981). In the years 1981-86, despite continued use of phenylamide plus protectant fungicide products, there was no consistent trend in the incidence of phenylamide-resistant strains and the proportion of isolates containing resistance was under 30% in every year except 1984 (Cooke, 1986). The situation changed in 1987; over 80% of isolates tested contained resistant strains. This high incidence of resistant strains was maintained in 1988 and 1989 and it was suggested that one of the major factors which led to selection for resistance was a succession of summers from 1985 to 1988 with weather very conducive to blight (Cooke, 1991). This paper reports the results of surveys of the occurrence of phenylamide-resistant *P. infestans* in Northern Ireland up to 1991 and of an investigation into the incidence of phenylamide-resistant strains within crops in 1990.

MATERIALS & METHODS

Sampling of potato crops for blight

Samples of infected potato foliage were collected by members of the Department of Agriculture's Potato Inspection Service, during the course of seed potato crop inspections. The 18 inspectors, each responsible for a specific geographical area, were asked to supply samples from at least three crops, preferably from cultivars typical of their regions. Foliage was collected from up to five sites within each crop and bulked together. Data on sample location, potato cultivar, fungicide usage and disease incidence were obtained for each sample and stored on a DATATRIEVE database.

In 1990, six crops with a foliage blight incidence of between 1 and 5% were selected for sampling in detail. Twenty single lesion samples (either one leaf or one stem) were collected from within each crop. Isolates from these were maintained and tested separately.

At the end of each season, Inspectors supplied details of fungicide usage for all seed potato crops in their areas.

Maintenance of isolates

Samples were maintained on detached leaves of glasshouse-grown potato plants cv. King Edward and sporangial/zoospore suspensions prepared as previously described (Cooke, 1986).

Tests for phenylamide resistance

Isolates were tested using the floating leaf disc technique (Cooke, 1986). Isolates were designated resistant if they sporulated on 100 mg metalaxyl/litre-treated discs and sensitive if they sporulated on untreated discs but not any metalaxyl-treated disc. Isolates which failed to grow on at least four out of six untreated discs were re-tested.

RESULTS

Incidence of phenylamide resistance

The overall proportion of isolates containing phenylamide-resistant strains of *P. infestans* for each of the years 1981-1991 is shown in Figure 1. In 1988, a peak of 90% of isolates containing resistance was reached, but since 1989 the proportion has declined to 42% in 1991.

When product usage on sampled crops was examined, it was found that those which had received phenylamide applications tended to have a greater proportion of isolates containing resistant strains, but that even crops where only protectant fungicides were used or where no fungicide had been applied often yielded resistant strains (Table 1). However, the decline in the proportion of isolates containing resistance was faster on non-phenylamide-treated crops.

FIGURE 1. The proportion of Northern Ireland isolates of *Phytophthora infestans* containing phenylamide-resistant strains, 1981-1991.

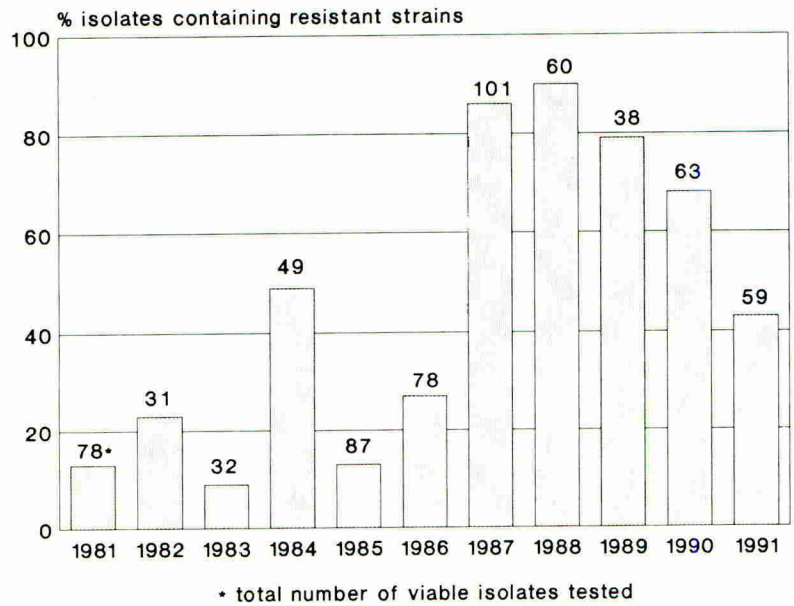


TABLE 1. Fungicide usage on potato crops sampled for *P. infestans* phenylamide resistance survey, 1988-91

Product type	% crops yielding isolates containing resistant strains*			
	1988	1989	1990	1991
none	100 (3)	67 (3)	60 (5)	100 (1)
unknown **	93 (15)	100 (3)	60 (10)	0 (0)
mancozeb	85 (26)	75 (28)	67 (42)	29 (38)
phenylamide	94 (16)	100 (4)	100 (6)	65 (20)
total	90 (60)	79 (38)	68 (63)	42 (59)

* total numbers of isolates tested are shown in brackets
** no usage of phenylamide-containing products

Investigation of the incidence of phenylamide-resistant *P. infestans* within crops, 1990

Of the six crops selected for detailed sampling in 1990, one was untreated at the time of sampling, three had received mancozeb only, and two had been treated with phenylamides (Table 2). Of these, Crop 5 had been sprayed once with metalaxyl plus mancozeb, whilst Crop 6 had received several applications of phenylamides (metalaxyl and oxadixyl).

TABLE 2. Incidence of phenylamide-resistant *P. infestans* within crops, 1990

Crop number	County	Potato cultivar	Fungicide treatment	No. of isolates tested	% isolates resistant
1	Down	Home Guard	none	20	10
2	Antrim	Home Guard	mancozeb	15	53
3	L'derry	Kerr's Pink	mancozeb	18	56
4	Antrim	Dunbar Standard	mancozeb	20	80
5	L'derry	King Edward	phenylamide	20	60
6	Antrim	Dundrod	phenylamide	19	95

Of the 20 single lesion samples collected from each crop, it proved possible to isolate from at least 15 and sometimes all 20 in each case. All the crops sampled proved to contain both resistant and sensitive strains, the lowest proportion of resistance being found in the unsprayed crop and the greatest in the crop which had received several phenylamide applications (Table 2). However, even one of the crops which had been treated with mancozeb alone yielded 80% resistant strains. After the lesions had been tested separately, those from each crop were bulked together and re-tested. All six bulk isolates gave a resistant result in the disc test; this was as expected since only 1% resistant sporangia are needed to give a resistant result in the disc test (Sozzi & Staub, 1987).

Usage of phenylamide-containing products

Potato Inspectors' estimates of usage of products containing phenylamides on seed potato crops are shown in Figure 2. Data for 1981 and 1982 are not available since in these years the agrochemical companies and the Department of Agriculture advised Northern Ireland

FIGURE. 2. Estimated seed potato crop area treated with phenylamides, 1983-1991.

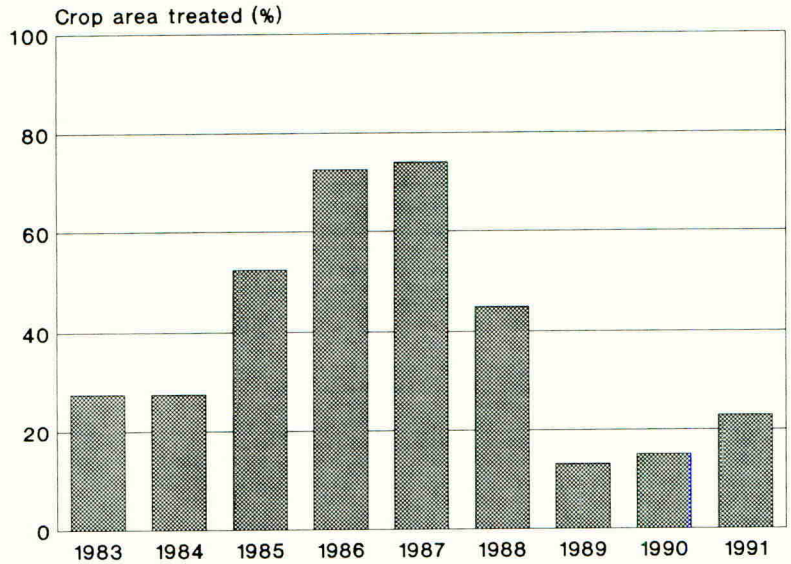
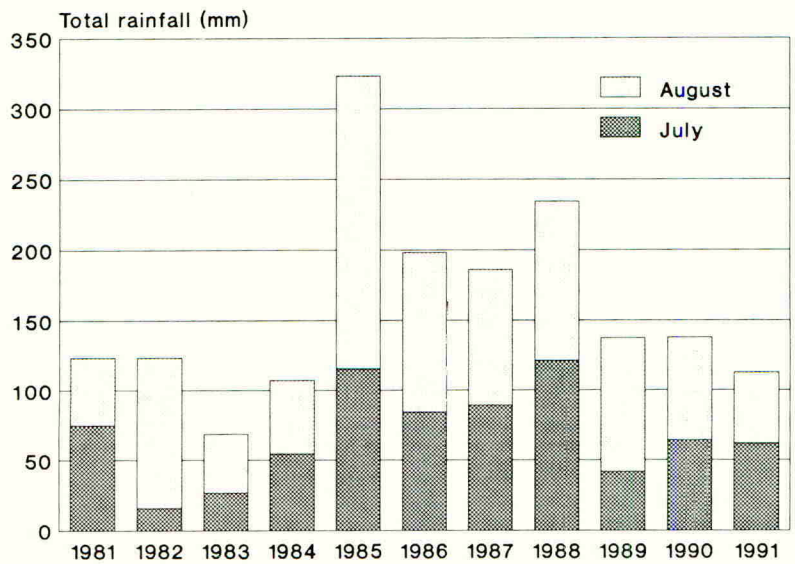


FIGURE 3. Total monthly rainfall for July-August, 1981-1991, Co. Londonderry.



growers not to use products containing phenylamides. Whereas in 1986-87 over 70% of the crop area was treated with phenylamides, after 1987 usage declined. In 1991, there was an increase in the reported area treated, although the proportion of growers using phenylamide-containing products for most of the season was slightly lower than in 1990 (10% compared with 15%). This may be due to a greater number of growers using one or two applications of phenylamides, rather than using them for most of the spray programme.

Meteorological data

In Northern Ireland, the first outbreaks of potato blight each year generally occur sporadically in mid-late June (once the night minimum temperature reaches *c.* 10°C) and infection builds up in July and August. Generally, rainfall and humidity in July and August have the greatest influence on foliage and tuber blight incidence, since at this time of the year night temperatures are not usually limiting. Rainfall data for July-August for the years 1981-91 are shown in Figure 3. The four seasons 1985-88 had unusually high rainfalls in July and August and in each of these years blight was widespread on crops in Northern Ireland. In contrast, the summers of 1989-91 had much lower rainfall and blight was generally well controlled.

DISCUSSION

The floating leaf disc test for phenylamide resistance is non-quantitative and detects resistant strains when they are present in an isolate at proportions greater than 1% (Sozzi & Staub, 1987). This may lead to an overestimation of resistance present in the field, if resistant strains are widespread, but at a low level. Using a semi-quantitative technique developed by Ciba-Geigy, only one of 10 Northern Ireland *P. infestans* isolates designated as resistant was confirmed to contain a mixture of resistant and sensitive sporangia, but, due to experimental variability, an isolate could not be identified as mixed unless it contained at least 30% sensitive sporangia (Walker, 1990). The investigation of the incidence of phenylamide-resistant *P. infestans* within crops reported here was based on the assumption that single lesion isolates should be clonal in origin and consist of all resistant or all sensitive strains. The results indicate that although both resistant and sensitive strains were found to occur together within crops, the resistant strains were in the majority in most cases and that taken together the mean proportion of resistant strains found in the single isolate tests (59%) was not far different from the overall result of the bulk isolate tests for that year (68%). There was no evidence to suggest that the overall results of the annual surveys were being severely distorted by a widespread but very sparse distribution of resistant strains.

After a dramatic increase in the proportion of isolates of *P. infestans* containing phenylamide-resistant strains in 1987-88 in Northern Ireland, the proportion has declined for three successive seasons (1989-1991). A similar trend has been observed in surveys in the Republic of Ireland (Dowley, L.J., personal communication). Two major factors *viz.* phenylamide usage and incidence of *P. infestans*, have interacted during this period to reduce the selection pressure in favour of resistance.

Usage of phenylamides on seed crops in Northern Ireland declined after 1987. Blight was widespread in phenylamide-treated crops in that season and growers were probably influenced by their perception of product performance. The Department of Agriculture also advised that the benefits of using phenylamides might not justify the additional cost in years when resistant strains were present in the great majority of *P. infestans* isolates tested (Cooke, 1990). Data for fungicide usage on ware crops are not available for most years. However, a Department of Agriculture survey of pesticide usage in 1990 indicated that 30% of the maincrop ware potato area was treated with phenylamides, a rather greater proportion than was estimated for seed crops in that year (15-20%), but that the great majority of crops received only one or two applications (Jess *et al.*, 1992).

In each of the years 1989-91, blight incidence in Northern Ireland was relatively low. The proportion of seed crops with more than 5% foliage infection at haulm destruction was been between 1 and 6%, in contrast to 1985 when it was >50%. Consequently, in each of these years only a minority of crops was treated with phenylamides and these were only exposed to a relatively small *P. infestans* population. Thus the selection pressure in favour of resistance was much reduced in contrast to the overwhelming pressure of the years 1985-88.

However, reduced pressure in favour of resistance alone will not bring about a decline in the proportion of resistant strains. If resistant and sensitive strains were identical in all respects save their response to phenylamides, the resistant proportion of the population might be expected to remain constant or decline very slowly as the resistant character was lost by random mutation. That a quite marked decline has occurred within three years suggests that the resistant strains may not be as fit as their sensitive counterparts. This may be due to a poorer capacity to survive the winter and produce primary infections the following season (Walker & Cooke, 1988, 1990). Nonetheless, it is clear that in years when foliage blight infection spreads rapidly, resistant strains will build up very quickly if application of phenylamides is widespread. The present moderate level of phenylamide usage is more likely to ensure that these fungicides continue to make some contribution to controlling potato blight than if they were once again to be applied to the majority of crops for most of the season.

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REFERENCES

Cooke, L.R. (1981) Resistance to metalaxyl in *Phytophthora infestans* in Northern Ireland. *1981 British Crop Protection Conference - Pests and Diseases*, **2**, 641-649.

Cooke, L.R. (1986) Acylalanine resistance in *Phytophthora infestans* in Northern Ireland. *1986 British Crop Protection Conference - Pests and Diseases*, **2**, 507-514.

Cooke, L.R. (1990) Potato blight control - the current position. *Agriculture in Northern Ireland*, **4** (8), 16-17.

Cooke, L.R. (1991) Current problems in potato blight control: the Northern Ireland experience. In: *Phytophthora*, J.A. Lucas, R.C. Shattock, D.S. Shaw and L.R. Cooke (Eds), Cambridge: Cambridge University Press, pp. 337-48.

Jess, S.; McCallion, T.; Kidd, S.B.L. (1992) *Pesticide usage in Northern Ireland - Survey Report 105 - Arable Crops 1990*, Department of Agriculture for Northern Ireland, in press.

Sozzi, D.; Staub, T. (1987) Accuracy of methods to monitor sensitivity of *Phytophthora infestans* to phenylamide fungicides. *Plant Disease*, **71**, 422-425.

Walker, A.S.L. (1990) *Comparison of the fitness of phenylamide-sensitive and phenylamide-resistant strains of* Phytophthora infestans. Ph.D. Thesis, The Queen's Univeristy of Belfast, pp. 50-53.

Walker, A.S.L.; Cooke, L.R. (1988) The survival of phenylamide-resistant strains of *Phytophthora infestans* in potato tubers. *Brighton Crop Protection Conference - Pests and Diseases 1988*, **1**, 353-358.

Walker, A.S.L.; Cooke, L.R. (1990) The survival of *Phytophthora infestans* in potato tubers - the role of phenylamide resistance. *Brighton Crop Protection Conference - Pests and Diseases 1990*, **3**, 1109-1114.

USE OF POLYMERASE CHAIN REACTION FOR THE DIAGNOSIS OF MBC RESISTANCE IN *BOTRYTIS CINEREA*

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ABSTRACT

In recent years resistance to methylbenzimidazole carbamate (MBC) in fungal pathogens has been attributed to single amino acid changes in the β-tubulin subunit. The majority of these changes are located between amino acids 100 and 300. Using conserved oligonucleotide primers encompassing this region we have been able to amplify, clone and sequence part of the β-tubulin gene from two *Botrytis cinerea* isolates with MBC resistant and sensitive phenotypes. A point mutation at amino acid 198, causing a change from glutamic acid to alanine, confered MBC resistance. We synthesised two oligonucleotide primers incorporating the point mutation for resistance and sensitivity. These allele specific oligonucleotides (ASO) were used in a nested primer PCR to determine the phenotype of several *B. cinerea* isolates of known sensitivity or resistance to MBC. The resistant and sensitive strands were successfully diagnosed by PCR amplification and Southern blot hybridisation.

INTRODUCTION

Resistance to benzimidazole fungicides was reported soon after these fungicides were released (Bollen & Scholten, 1971; Delp, 1980). Extensive studies concerning the mode of action of methyl benzimidazole carbamate (MBC) have been closely paralleled by those on the mechanism of resistance. MBC has been shown to bind preferentially to β-tubulin (Davidse, 1973; Davidse & Flach, 1977) and small changes in the β-tubulin protein result in reduced affinity for the fungicide (Sheir-Neiss *et al* ., 1978).

The study of fungal resistance has been greatly enhanced by the isolation of laboratory mutants resistant to these mitotic inhibitors (van Tuyl, 1975; Orbach *et al* ., 1986). Cloning and sequencing of β-tubulin genes from these isolates has enabled the cause of MBC resistance in many fungal strains to be located as point mutations in the β-tubulin gene and which result in single amino acid changes (Thomas *et al* ., 1985; Orbach *et al* ., 1986; Jung *et al* ., 1987) and which influence the electrophoretic properties of the protein subunit (Shier-Neiss *et al* ., 1976 & 1978; May *et al* ., 1985 & 1987).

In this paper we discuss the discovery of the point mutation responsible for MBC resistance in *B. cinerea* isolates and its diagnosis by use of the polymerase chain reaction (PCR) adopting allele-specific oligonucleotide primers (ASO).

MATERIALS AND METHODS

Strains and plasmids

B.cinerea isolates used in this study are listed in Table 1. The EC_{50} and phenotypes were predetermined by growth on DYP agar (Groves *et al.*, 1988) in the presence of MBC. Isolate K1145, resistant to MBC (MBCR), and PC9385S, sensitive to MBC (MBCS) were used as a source of genomic DNA for cloning and sequencing of the β-tubulin gene. Genomic DNA was extracted from 3 day old mycelial cultures according to Stevens *et al*. (1982).

The bacterial strain *Escherichia coli* DH5α (BRL, Life Technologies, Gaithersberg, Md.) cultured on or in Luria-Bertani medium (Maniatis *et al.*, 1982) was used for transformation and isolation of plasmids.

Amplification of β-tubulin target sequence by PCR

The β-tubulin gene segment from *B. cinerea* genomic DNA (1μg) was amplified using primers β–101 and β-293 (see below) in 100μl reaction volumes containing 200μM of each deoxynucleotide triphosphate (dNTPs),400pM of each primer , 2.5units of *Ampli* Taq® polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), 50mM KCl, 10mM Tris-HCl (pH 8.3), 1.5mM $MgCl_2$, and 0.01% gelatine.The procedure was as follows: denaturation at 95°C for 2 min; annealing at 55°C for 2min; extension at 72°C for 3 min for 30 cycles with a final extension at 72°C for 7 min. Reaction mixture (8μl) was loaded on to 1.5% (w/v) agarose gels containing ethidium bromide and run at 50V. The amplified DNA was visualised by u.v. light and the position of bands compared to a standard λ ladder .

Diagnosis of resistance using ASO in a nested primer system

Reaction mixtures (50μl) were prepared in duplicate containing approximately 100ng of amplified β-tubulin (1μl of PCR product), primer β-293 and tailed primer R or tailed primer S. The samples were amplified using the following conditions: denaturation at 95°C for 1.5 min, annealing at 62°C for 1.5 min, extension at 72°C for 3 min - for 2 cycles followed by the same process with an annealing temperature of 67°C for 28 cycles. The products were run on 1.5% agarose gels as previously described.

Cloning and sequencing

The recessed ends of the amplified β-tubulin gene segment were filled using the Klenow fragment of *E. coli* DNA polymerase I, followed by the addition of phosphates using polynucleotide kinase. The amplified gene fragment was ligated to dephosphorylated (calf intestinal phosphatase) pBluescript SK_+ (Stratagene, La Jolla, Ca) in the presence of T4 ligase (BRL) and blunt end ligation buffer . The mixture was used to transform *E. coli* DH5α. Transformants, identified by blue/white selection, were recultured and plasmid DNA isolated and screened for the presence of the β-tubulin insert by restriction digest using BamH1 and Hind III. All methods described above are according to Maniatis *et al* . (1982) and Sambrook *et al* . (1989).

Plasmid DNA was isolated from selected clones and nucleotide sequence analysis performed using the dideoxy chain termination method (Sanger *et al* .,1977) with the Sequenase® kit (U.S. Biochemicals, Cleveland, Ohio).

RESULTS AND DISCUSSION

Amplification of part of the β-tubulin gene from *B. cinerea* and its subsequent cloning and sequencing.

The majority of mutations causing MBC resistance have been located between amino acid residue 100 and 300 of the β-subunit (reviewed by Leroux, 1991). Primers β-101 and β-293 were designed in highly conserved regions of the β-tubulin gene flanking this area of interest based upon the β-tubulin amino acid sequence comparisons outlined by Orbach *et al.*. (1986). Primer oligonucleotide sequences are shown in Figure 1.

Primers and Probe

Amplification of β-tubulin gene

Primers: β-101: TGG GCT AAA GGT CAC TAC AC
β-293: CAT TTG TTG TGT TGT TAA TTC TGG

Diagnosis of MBC resistance using ASO primers

Primer S: G AGA ACT CTG ACG A
Primer R: G AGA ACT CTG ACG C

Tailed Primer S: GCT GGC CAA CTG AGA ACT CTG ACG A
Tailed Primer R: GCT GGC CAA CTG AGA ACT CTG ACG C

Southern blotting R- probe:

TCT GAC GCG ACC TTC TGT

FIGURE 1. Nucleotide sequences for primers and probe.

Amplification by PCR of genomic DNA from *B. cinerea* K1145 (MBCR) and PC9385S (MBCS) resulted in a fragment 579bp in size as predicted for these chosen primer positions. These fragments were cloned into pBluescript and 30 positive DH5α transformants which grew as white colonies were selected. Restriction digest with Hind III and Bam HI revealed 8 clones with plasmids containing the β-tubulin insert. Three transformants containing plasmids with inserts from K1145 (MBCR) and one from PC9385S (MBCS) were chosen. From these the plasmids were purified on a caesium chloride gradient (Maniatis *et al* ., 1982) and sequenced. The nucleotide sequence for *B. cinerea* K1145 is shown in Figure 2.

To verify that the PCR fragment was indeed part of the *B. cinerea* β-tubulin gene, we compared the predicted protein sequence with published data for several other fungal β-tubulin genes (within the region amino acid 100 - 300). The amino acid sequence for the *B. cinerea* β-tubulin is highly homologous to *Neurospora crassa* (FGSC 3460) 98% (Orbach *et al* ., 1986), *Schizosaccharomyces pombe* 83% (Hirako *et al* ., 1984), *Aspergillus nidulans* 97% (May *et al* ., 1987) and *Saccharomyces cerevisiae* 84% (Neff *et al* ., 1983) (Figure 3).

Mapping of the point mutation responsible for MBC resistance in *B. cinerea.*

Sequence comparisons of the target DNA from *B. cinerea* K1145 (MBCR) and PC9385S (MBCS) revealed a single transversion from A to C at nucleotide 295 of the K1145

```
                                                *30
TGG GCG AAG GGT CAT TAC ACT GAG GGT GCT GAG CTT GTC GAC CAA GTT CTT GAT
Trp Ala Lys Gly His Tyr Thr Glu Gly Ala Glu Leu Val Asp Gln Val Leu Asp
101                                     110
      *60                                           *90
GTT GTC CGT CGT GAA GCT GAA GGC TGT GAC TGC CTT CAA GGA TTC CAA ATT ACC
Val Val Arg Arg Glu Ala Glu Gly Cys Asp Cys Leu Gln Gly Phe Gln Ile Thr
    120                                         130
              *120                                          *150
CAC TCT CTC GGT GGT GGA ACT GGT GCC GGT ATG GGT ACG CTT TTG ATC TCC AAG
His Ser Leu Gly Gly Gly Thr Gly Ala Gly Met Gly Thr Leu Leu Ile Ser Lys
            140                                         150
                       *180                                         *210
ATC CGC GAG GAG TTC CCA GAT CGT ATG ATG GCT ACC TTC TCC GTC GTC CCA TCG
Ile Arg Glu Glu Phe Pro Asp Arg Met Met Ala Thr Phe Ser Val Val Pro Ser
                       160                                         170
                                  *240                                          *270
CCA AAG GTT TCC GAT ACC GTT GTC GAG CCA TAT AAC GCA ACT CTC TCT GTC CAT
Pro Lys Val Ser Asp Thr Val Val Glu Pro Tyr Asn Ala Thr Leu Ser Val His
                                  180                                         190
                                              *300
CAA TTG GTT GAG AAC TCT GAC GCG ACC TTC TGT ATC GAT AAC GAG GCT CTT TAC
Gln Leu Val Glu Asn Ser Asp Ala Thr Phe Cys Ile Asp Asn Glu Ala Leu Tyr
                                        200
      *330                                          *360
GAT ATT TGC ATG AGA ACC TTG AAG CTC AGC AAC CCA TCT TAC GGA GAT CTT AAC
Asp Ile Cys Met Arg Thr Leu Lys Leu Ser Asn Pro Ser Tyr Gly Asp Leu Asn
    210                                         220
              *390                                          *420
CAC TTG GTT TCC GCC GTC ATG TCC GGT GTT ACC ACC TGT CTC CGT TTC CCT GGT
His Leu Val Ser Ala Val Met Ser Gly Val Thr Thr Cys Leu Arg Phe Pro Gly
            230                                         240
                       *450                                         *480
CAA CTT AAC TCA GAT CTC CGA AAG TTG GCT GTT AAC ATG GTT CCA TTC CCC CGT
Gln Leu Asn Ser Asp Leu Arg Lys Leu Ala Val Asn Met Val Pro Phe Pro Arg
                       250                                         260
                                  *510                                          *540
CTC CAT TTC TTC ATG GTT GGA TTT GCT CCT TTG ACC AGT CGT GGC GCA CAC TCT
Leu His Phe Phe Met Val Gly Phe Ala Pro Leu Thr Ser Arg Gly Ala His Ser
                                  270                                         280
                                              *570
TTC CGT GCT GTC ACC GTT CCC GAA CTG ACA CAA CAG ATG
Phe Arg Ala Val Thr Val Pro Glu Leu Thr Gln Gln Met
                                        290
```

FIGURE 2. DNA sequence for part of the β-tubulin gene from *B. cinerea* K1145 (MBCR). The nucleotide sequence is numbered above and relates to the cloned β-tubulin gene fragment. The amino acids are numbered below the sequence and relate to the position of the residue in relation to the whole β-tubulin sequence.

```
            *101        *111        *121        *131        *141
B.cK        WAKGHYTEGA ELVDQVLDVV RREAEGCDCL QGFQITHSLG GGTGAGMGTL
B.cP
N.crassa 3460
A.nidulans                 N V
S.cerevisiae               S M  I          S                 S
S.pombe                  AVA L           A  A       L        S


            *151        *161        *171        *181        *191
B.cK        LISKIREEFP DRMMATFSVV PSPKVSDTVV EPYNATLSVH QLVENSDATF
B.cP                                                          E
N.c                                                           E
A.n                                                         H E
S.c          F   K  L           L     T                    H E
S.p          L      Y           A  A  S              M        E


            *201        *211        *221        *231        *241
B.cK        CIDNEALYDI CMRTLKLSNP SYGDLNHLVS AVMSGVTTCL RFPGQLNSDL
B.cP
N.c                                                VS
A.n
S.c                     Q     NQ        N    S       S   Y
S.p               SS  IAN   IKS    D           A    S      E


            *251        *261        *271        *281        *291
B.cK        RKLAVNMVPF PRLHFFMVGF APLTSRGAHS FRAVTVPELT QQM
B.cP
N.c                                       H     S
A.n           W                           Y     S
S.c               L             Y     AI SQ    SL
S.p                                   AAI SS   Q  S
```

FIGURE 3. Comparison of the amino acid sequence for β-tubulin from residue 101 to 193 of *B. cinerea* (K1145 and Pc9385S) with those of other fungal β-tubulin genes. Only residues varying from *B. cinerea* are shown. Numbers at the top of the sequence denote the position of the amino acid residue in relation to the whole β-tubulin sequence.

β-tubulin sequence. This resulted in a glutamic acid to alanine change at position 198 (numbered in relation to the whole β-tubulin subunit).

As stated earlier the majority of mutations resulting in MBC resistance have been mapped between amino acids 100 and 300 of the β-tubulin subunit. Orbach *et al* . (1986) localised resistance as a phenylalanine to tyrosine change at amino acid 167 for *N. crassa*. In *S. cerevisiae* a change from arginine to histidine at amino acid 241 was responsible for benomyl resistance (Jung *et al* ., 1985). More recently MBC resistance has been attributed to point mutations at amino acid 198. For *Venturia inaequalis* a change from glutamate to alanine (Leroux, 1991) and for *N. crassa* F914 glutamic acid to glycine substitution resulted in MBC resistance (Fujimura *et al* ., 1992). We concluded that the mutation at position 198 for *B. cinerea* K1145 is responsible for its MBC resistance.

Development of a PCR diagnostic test.

We proceeded on the basis that allele-specific oligonucleotide primers (ASO) (incorporating the base change for either MBC resistance or sensitivity) could be used in conjunction with primer β-293 as a diagnostic assay, able to discriminate between the

genotypes of *B. cinerea* isolates.The assay would involve selective amplification of β-tubulin with corresponding MBC sensitive or resistant nucleotide sequences.

Originally the primers R and S outlined above were synthesised (see Figure 1). It was assumed a PCR product of approximately 300bp would be amplified. This was achieved and a PCR fragment of 300bp obtained, but without selectivity. The major cause was thought to be the non-specific priming ability of *Ampli* Taq® polymerase. To overcome the problem, primers were designed with a tail region which was unrelated to the tubulin sequence within the area of the mutation (see Figure 1.) . The use of tailed primers has been outlined by Jefferies *et al* . (1991). However in the authors' technique a third primer was added after the initial PCR cycles. In the technique described below the same increase in specificity was achieved by altering the annealing temperature after the first two cycles.

The assay was extremely successful when using a nested primer system. A PCR fragment of 310bp (as predicted) was amplified with both tailed primers-S and R. The results (Table 1) indicated amplification of the β-tubulin sequence by the S-primer, (but not the R-primer) for *B. cinerea* B4, A19 and PC9385S. These isolates had been previously characterised as MBC sensitive. Isolates Pc9385R, K1145, and 1805R were amplified using the R-primer, (but not by the S-primer). It was concluded that they were MBC resistant. Poison plate analysis confirmed these cultures were indeed resistant. Amplification of β-tubulin from an intermediate isolate GB111/74 occurs with both tailed primer S and tailed primer R. In this case an alternative point mutation or another mechanism of resistance may be in operation. Koenraadt *et al* . (1991) claimed that a mutation at amino acid 198 from glutamate to glycine caused intermediate resistance in an *V. inaequalis* isolate.

TABLE 1. Screening of *B. cinerea* isolates for MBC resistance.

Isolate	EC_{50} µg.ml^{-1}	Phenotype	Presence of resistant sequence PCR primer S	PCR primer R	Southern blot R-probe
PC9385R	775	Resistant	-	+	+
K1145	733	Resistant	-	+	+
1805R(b)	425	Resistant	-	+	+
GB 111/74	17.8	Intermediate	+	+	not tested
PC9385S	0.066	Sensitive	+	-	-
A19	0.055	Sensitive	+	-	not tested
B4	0.075	Sensitive	+	-	-

EC_{50} reduction in colony diameter by 50%
+ indicates strong amplification by PCR and /or hybridisation by Southern blotting
- indicates no visible amplification by PCR and/or hybridisation by Southern blotting

To confirm the results above, Southern blots (Southern, 1975) of the amplified β-tubulin target sequences from five of the *B. cinerea* isolates were hybridised with an 18-mer oligonucleotide probe (sequence corresponding to MBC resistance (Figure 1) with the mutation placed centrally to aid hybridisation. The probe was labelled with [γ^{32}P] ATP (Maniatis *et al* ., 1982). Autoradiographs showed that the probe bound specifically to the corresponding resistant sequences (Table 1). It was concluded that the diagnostic PCR was able to discriminate between the point mutations for resistance and sensitivity.

Possible use of PCR as a rapid diagnostic test

We found that it was possible to assess MBC resistance or sensitivity within 48-72 hours. The test is far more rapid than standard poison plate assays which can take up to three weeks and rely on clean cultures. The ASO-PCR is remarkably sensitive, with the potential to amplify as little as 1ng of target DNA. We believe that less fungal material will be required for this method than for those based on monoclonal antibodies (Martin *et al.*, 1992). PCR will detect genotypic rather than phenotypic resistance, which is potentially more useful when diagnosing resistance in a heterokaryotic fungus such as *B. cinerea*.

Thus our attempts amplify specifically R and S β-tubulin sequences from genomic DNA from *B. cinerea* have proven as yet unsuccessful, whereas we have succeeded using a nested primer system, whereby the 579bp fragment is amplified non-specifically , followed by a specific detection step for the 310bp fragment. Similar results have been reported for other diagnostic tests using PCR. Amplification of target sites is influenced by the cleanness of genomic DNA preparations (A. Daly, unpublished). It may be concluded that specificity of the assay will rely on the continued use of a nested primer system. This has potential for development into a sensitive diagnostic test, and future work will attempt to increase the ease and throughput of the assay.

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REFERENCES

Bollen, G.J.; Scholten, G.(1971) Acquired resistance to benomyl and some other systemic fungicides in a strain of *Botrytis cinerea* in cyclamen. *Netherlands Journal of Plant Pathology* , **77**, 83-90

Davidse, L.C. (1973) Antimitotic activity of methyl benzimidazole carbamate (MBC) in *Aspergillus nidulans*. *Pesticide Biochemistry and Physiology*, **3**, 317-325

Davidse, L.C.; Flach, W. (1977) Differential binding of methyl benzimidazol-2-yl carbamate to fungal tubulin as a mechanism of resistance to this anti-mitotic agent in mutant strains of *Aspergillus nidulans*. *Journal of Cell Biology* ,**72**, 174-193.

Delp, C.J. (1980) Coping with resistance to plant disease control agents. *Plant Disease*, **64**, 652-657.

Fujimura, M.; Oeda, K.; Inoue, H.; Kato, T. (1992) The single amino acid substitution in the beta tubulin gene of *Neurospora crassa* confers both carbendazim resistance and diethofencarb sensitivity. *Current Genetics* , **21**, 399-404

Groves, J.D.; Fox, R.T.V.; Baldwin, B.C. (1988) Modes of action of carbendazim and ethyl *N* -(3,5-dichlorophenyl) carbamate on field isolates of *Botrytis cinerea*.. *Brighton Crop Protection Conference- Pest and Diseases 1988*, **1**, 397-402

Hiraoka, Y.; Toda, T. ; Yanagida, M. (1984) The NDA3 gene of fission yeast encodes β-tubulin: a cold-sensitive nda3 mutation reversibly blocks spindle formation and chromosome movement in mitosis. *Cell* , **39** , 349-358

Koenraadt, H.; Somerville, S.C.; Jones, A.L.(1991) Molecular characterisation of the β-tubulin gene from benomyl sensitive and benomyl resistant field strains of *Venturia inaequalis*. *Abstracts of the American Chemical Society , April 1991*, AGRO 87

Jeffreys, A.J.; MacLeod, A.; Tamaki, K.; Neil, D.L.; Monckton D.G. (1991) Minisatellite repeat coding as a digital approach to DNA typing. *Nature*, **354**, 204-209

Jung, M.K.; Dunne, P.W.; Suen, I.-H. ; Oakley, B.R. (1987) Sequence alterations in β-tubulin mutations of *Aspergillus nidulans*. *Journal of Cell Biology*, **105**, Abstracts, 277a.

Leroux, P. (1991) Résistance des Champignons phytopathogénes aux fongicides. *Phytoma - La Défenses des végétaux*, **434**, 20-26

Maniatis, T.; Fritsch, E.F. ; Sambrook.J. (1982) *Molecular cloning: a laboratory manual.* Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y.

Martin, L.-A.; Fox, R.T.V.; Baldwin, B.C. (1992) Rapid methods for detection of MBC resistance in fungi: I. Immunological approach. *Proceedings of 10th International Reinhardsbrunn Symposium.* (in press)

May, G.S.; Gambino, J.; Weatherbee, J.A.; Morris, N.R. (1985) Identification and functional analysis of beta-tubulin genes by site specific integrative transformation in *Aspergillus nidulans*. *Journal of Cell Biology*, **101**,712-719

May, G.S.; Tsang, M.L.S.; Smith, H.; Fidel, S.; Morris, N.R. (1987) *Aspergillus nidulans* β-tubulin genes are unusually divergent. *Gene*, **55**, 231-243

Neff, N.F.; Thomas, J.H.; Grisafi, P.; Botstein, D. (1983) Isolation of the β-tubulin gene from yeast and a demonstration of its essential function *in vivo*. *Cell*, **33**, 211-219

Orbach, M.J.; Porro, E.B.; Yanofsky, C. (1986) Cloning and characterization of the gene for β-tubulin from a benomyl-resistant mutant of *Neurospora crassa*, and its use as a dominant selectable marker. *Molecular and Cellular Biology*, **6**, 2452-2461.

Sambrook, J.; Fritsch, E.F.; Maniatis, T. (1989) Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y.

Sanger, F.; Nicklen S.; Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Science. USA* ,**74**, 5463-5467

Sheir-Neiss, G.; Nardi, R.V.; Gealt, M.A.; Morris, N.R. (1976) Tubulin like protein from *Aspergillus nidulans. Biochemical and Biophysical Research Communications*, **69**, 285-290

Sheir-Neiss, G.; Lai, M.; Morris, N.R. (1978) Identification of a gene for β-tubulin in *Aspergillus nidulans. Cell*, **15**, 639-647

Southern, E.M. (1975) Detection of specific sequences among DNA fragments seperated by gel electrophoresis. *Journal of Molecular Biology*, **98**, 503-517

Stevens, J.N.; Metzenberg, R.L. (1982) An easy method for preparing *Neurospora* DNA. *Neurospora News Letters*, **29**, 27-28

Thomas, J.H.; Neff, N.; Botstein, D. (1985) Isolation and characterization of mutations in the β-tubulin gene of *Saccharomyces cerevisiae. Genetics*, **112**, 715-734

van Tuyl, J.M. (1975) Genetic aspects of acquired resistance to benomyl and thiabendazole in a number of fungi. *Mededelingen van de Faculteit Landbouwwetenschappen Rijksuniversiteit Gent,* **40**, 691-697

EFFECT OF MORPHOLINE-LIKE FUNGICIDES ON GROWTH AND STEROL COMPOSITION OF A WILD-TYPE STRAIN AND A STEROL MUTANT OF *USTILAGO MAYDIS* DEFECTIVE IN STEROL $\Delta^8 \rightarrow \Delta^7$ ISOMERASE ACTIVITY

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ABSTRACT

The effect of tridemorph, fenpropimorph and fenpropidin on the growth and sterol content of a *Ustilago maydis* wild-type strain and a sterol mutant, defective in sterol $\Delta^8 \rightarrow \Delta^7$ isomerization, has been examined. The results, which are in agreement with previous findings, indicated that tridemorph primarily affects sterol $\Delta^8 \rightarrow \Delta^7$ isomerization, whereas fenpropidin was more active against sterol Δ^{14} reduction. In contrast, fenpropimorph appeared to be inhibitory against both these enzyme activities. Inhibition of growth by fenpropimorph and fenpropidin, in both the wild-type and the mutant, coincided with the accumulation of the Δ^{14} sterol, ignosterol. Although tridemorph inhibited growth of the mutant by 45%, its sterol composition was unaffected. These results suggests that the fungicidal effectiveness of fenpropidin and fenpropimorph is associated with the accumulation of ignosterol, while inhibition of growth by tridemorph appears to result, in part, from additional effects on processes unrelated to sterol biosynthesis. Exposure of the wild-type and the sterol mutant to tridemorph or fenpropimorph also led to an increase in the degree of unsaturation of fatty acids.

INTRODUCTION

The outstanding success of sterol biosynthesis inhibitors (SBIs) as fungicides has been qualified in recent years by the appearance of resistant fungal strains in the field (Koller & Scheinpflug, 1987). This problem is particularly evident with inhibitors of sterol C-14 demethylase (DMIs). In contrast, the morpholine-type fungicides, while having a narrower spectrum of fungicidal activity, seem to be less beset by resistance problems (Brent, 1988). Unlike DMIs, which act at a single site, morpholines are generally considered to have a dual mode of action and inhibit both sterol $\Delta^8 \rightarrow \Delta^7$ isomerization and sterol Δ^{14} reduction to different degrees, depending on the structure of the inhibitor and the species of fungus under investigation (Mercer, 1991).

Fungal strains deficient in sterol biosynthesis are,

paradoxically, viable and able to accumulate abnormal sterols which are not normally found in the wild-type. In a recent study we isolated a number of polyene-resistant mutants of *Ustilago maydis*, defective in ergosterol biosynthesis (James *et al*,. 1992). Among these mutants was a pimaricin-resistant isolate which contained no ergosterol but instead accumulated Δ^8 sterols. This isolate, which is presumed to be defective in sterol $\Delta^8 \rightarrow \Delta^7$ isomerase activity, has been used to study the mode of action of morpholine fungicides and to identify potential sources of resistance to these inhibitors. We reasoned that fungicides that primarily affect sterol $\Delta^8 \rightarrow \Delta^7$ isomerization should be ineffective against this mutant, while those that inhibit sterol Δ^{14} reduction or both enzymatic steps should retain their activity because $\Delta^8 \rightarrow \Delta^7$ isomerization follows Δ^{14} reduction in ergosterol biosynthesis. However, care is needed in interpreting the effects of these inhibitors on fungal growth because any biochemical compensation which permits growth and allows Δ^8 sterols to substitute for ergosterol might also allow other sterols, including Δ^{14} sterols, to functionally replace ergosterol.

STEROL CONTENT AND GROWTH OF *U. MAYDIS* PIMARICIN-RESISTANT MUTANT

Characterization of the pimaricin-resistant mutant, P51, has been described previously (James *et al*., 1992). Unlike the wild-type, IMI103761, this mutant contained no detectable ergosterol, but instead accumulated the Δ^8 sterols, ergost-8-enol, ergosta-8,22-dienol, ergosta-5,8,22-trienol and fecosterol (ergosta-8,24(28)-dienol). The growth of the mutant was reduced as compared to the wild-type. Sporidia produced by this mutant were morphologically abnormal and often grew as multicellular clumps of cells. However, subculturing the mutant during the early stages of log growth enabled growth rates of between 38-58 % of the wild-type to be attained. Cultures of the mutant grown in this way contained many single cells along with the characteristic cell clumps. However, no changes in the type of sterols formed by the mutant was observed after continuous subculture and cultures grown in this way were used in the experiments described below.

SENSITIVITY OF *U.MAYDIS* WILD-TYPE AND STEROL MUTANT TO MORPHOLINE-LIKE FUNGICIDES

Tridemorph, fenpropimorph and fenpropidin were potent inhibitors of growth of the *U. maydis* wild-type (Figures 1A,1C,1E). In agreement with a recent study (Girling 1991), fenpropidin was significantly less active than the other two inhibitors. As compared to the wild-type, the mutant was appreciably less sensitive to tridemorph (Figure 1B) but comparable in growth after exposure to inhibitory concentrations of fenpropimorph (Figure 1D). Surprisingly, the sterol mutant was more sensitive to fenpropidin than the wild-type (Figure 1F).

STEROL COMPOSITION OF *U. MAYDIS* WILD-TYPE AND STEROL MUTANT FOLLOWING TREATMENT WITH MORPHOLINE-LIKE FUNGICIDES

Ergosterol was the predominant sterol in the wild-type. Exposure to all three inhibitors depleted ergosterol and this was closely associated with a reduction in growth (Figures 1A,1C,1E). Increasing the concentration of tridemorph led to a progressive increase in ergost-8-enol and ergosta-8,22-dienol in the wild-type, whereas within the same concentration range, tridemorph did not alter the sterol composition of the mutant, even though growth was reduced by up to 45 percent (Figure 1B). In contrast, ergost-8-enol and ergosta-8,22-dienol in the wild-type initially increased with increasing concentrations of fenpropimorph and then declined at higher concentrations. This decline in Δ^8 sterols was associated with an increase in the Δ^{14} sterol, ignosterol. Δ^8 sterols did not accumulate in the wild-type following exposure to fenpropidin. In this case, depletion in ergosterol was associated with an accumulation of ignosterol (Figure 1E). Inhibition of growth of the mutant by either fenpropimorph or fenpropidin was correlated with a reduction in ergost-8-enol and ergosta-8,22-dienol and an increase in ignosterol (Figure 1D,1F).

EFFECT OF INHIBITORS ON FATTY ACID COMPOSITION OF POLAR LIPIDS FROM *U.MAYDIS* WILD-TYPE AND STEROL MUTANT

The effect of tridemorph and fenpropimorph, at selected concentrations, on fatty acids derived from polar lipids of the wild-type and the mutant is shown in Table 1. Both strains exhibited a pronounced increase in the degree of fatty acid unsaturation following treatment with the fungicides.

DISCUSSION

The results presented here demonstrate the potential value of sterol biosynthesis mutants for unravelling the mode of action of SBIs. Considering the potency of SBIs, it is surprising that mutants defective in sterol biosynthesis are viable and able to grow, albeit, at reduced rates. The sterol mutant used in this

FIGURE 1. Effect of tridemorph (A,B), fenpropimorph (C,D) and fenpropidin (E,F) on growth (———) and sterol composition of the *U. maydis* wild-type (A,C,E) or the C-8 sterol isomerase deficient mutant (B,D,F). ergosterol (-----), ergost-8-enol and ergosta-8,22-dienol (·····); ignosterol and fecosterol (– — –). The amounts of ergosterol, ergost-8-enol and ergosta-8,22-dienol were calculated as described previously (James *et al.*, 1992). Due to difficulties in separating ignosterol from fecosterol by capillary GC, UV absorbtion of the crude sterol extracts at 250 nm was used to detect the presence of ignosterol (molar extinction coefficient, 18000) (Baloch *et al.*, 1984). The fungicide treatments at which UV absorption due to ignosterol was first detected are marked with an asterisk.

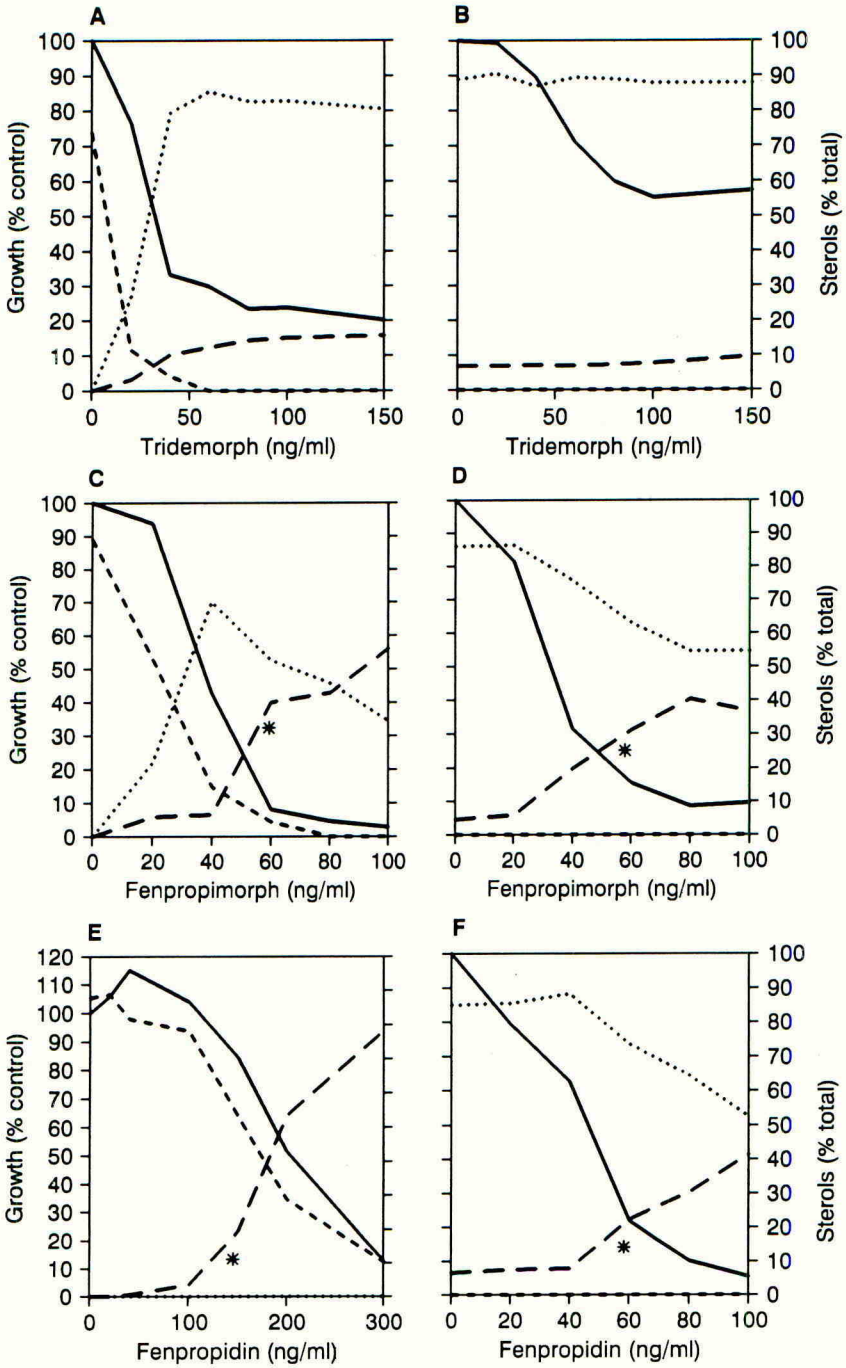
A
B
C
D
E
F
Growth (% control)
Sterols (% total)
Tridemorph (ng/ml)
Fenpropimorph (ng/ml)
Fenpropidin (ng/ml)
*

TABLE 1. Fatty acid composition of *U. maydis* wild-type and sterol mutant following treatment with tridemorph or fenpropimorph. Analysis of polar lipids was as described by Carter *et al.*, (1989).

U. maydis strain	Treatment (% inhibition)	Fatty acid composition				
		16.0	18.0	18.1	18:2	18:2/ 18.3
IMI103761	Untreated (0%)	18.1	2.0	52.0	21.5	0.41
IMI103761	Tridemorph, 100 ng/ml(75%)	20.5	1.4	7.2	62.3	8.65
IMI103761	Fenpropimorph, 40 ng/ml(60%)	26.1	2.4	12.7	53.3	4.19
P51	Untreated (0%)	21.6	2.4	29.6	40.7	1.37
P51	Tridemorph, 100 ng/ml(45%)	23.2	1.0	6.1	62.4	10.23
P51	Fenpropimorph, 40 ng/ml(70%)	21.8	1.0	6.9	61.6	8.93

study, as with other fungal sterol mutants (Garber *et al.*, 1989), is defective in membrane function. Such a membrane impairment, caused by ergosterol deficiency, could prevent biotrophic fungi from acquiring essential nutrients and metabolites from the host plant during infection and this may contribute to the effectiveness of these inhibitors as fungicides.

These data support the view that although inhibition of sterol $\Delta^{8}\rightarrow\Delta^{7}$ isomerase contributes to the action of morpholine fungicides, inhibition of sterol Δ^{14} reductase may be more effective in preventing fungal growth. Indeed, it is possible that ignosterol is toxic to fungi. However, this seems unlikely since a *Neurospora crassa* sterol mutant which accumulates Δ^{14} sterols, including ignosterol, can grow and is viable (Ellis *et al.*, 1991).

The increase in unsaturated fatty acids following treatment with either tridemorph or fenpropimorph, is similar to those reported in *U. maydis* exposed to the DMI, triarimol (Ragsdale 1975). This change in fatty acid composition of treated cells may compensate for the altered sterol content of cell membranes. However, this pattern of fatty acid saturation is likely to result from impaired growth rather than from a reduction in ergosterol content (Weete *et al.*, 1991).

Finally these results confirm a dual site of action for the morpholine fungicides in sterol biosynthesis and suggests that, at least in the case of tridemorph, another site of action unconnected with sterol biosynthesis exists. The multiple action of this type of inhibitor may, thus, explain the observed lack of resistance to morpholines observed in the field.

REFERENCES

Baloch, R.I.; Mercer, E.I.; Wiggins, T.E.; Baldwin, B.C. (1984) Inhibition of ergosterol biosynthesis in *Saccharomyces cerevisiae* and *Ustilago maydis* by tridemorph, fenpropimorph and fenpropidin. *Phytochemistry*, **23,** 2219-2226.

Brent, K.J. (1988) Resistance experiences in Europe. In *Fungicide Resistance in North America*, C.J. Delph (Ed), St Paul, APS Press, pp 19-27.

Carter, G.A.; Kendall, S.J.; Burden, R.S.; James, C.S.; Clarke, T. (1989) The lipid compositions of two isolates of *Cladosporium cucumerinum* do not explain their differences in sensitivity to fungicides which inhibit sterol biosynthesis. *Pesticide Science*, **26,** 181-192.

Ellis, S.W.; Rose, M.E.; Grindle, M. (1991) Identification of a sterol mutant of *Neurospora crassa* deficient in $\Delta^{14,15}$ reductase activity. *Journal of General Microbiology*, **137,** 2627-2630.

Garber, R.F.; Copple, D.M.; Kennedy, B.K.; Vidal, M.; Bard, M. (1989) The yeast gene *ERG6* is required for normal membrane function but is not essential for biosynthesis of the cell-cycle-sparking function. *Molecular and Cellular Biology*, **9,** 3447-3456.

Girling, I.J. (1991) The mode of action of morpholine fungicides and their mechanism of selectivity between fungal species. *PhD Thesis,* University of Bristol.

James, C.S.; Burden, R.S.; Loeffler, R.S.T.; Hargreaves, J.A. (1992) Isolation and characterization of polyene-resistant mutants from the maize smut pathogen, *Ustilago maydis*, defective in ergosterol biosynthesis. *Journal of General Microbiology*, **138,** 1437-1443.

Koller, W; Scheinpflug, H. (1987) Fungal resistance to sterol biosynthesis inhibitors: A new challenge. *Plant Disease*, **71,** 1066-1074.

Mercer, E.I. (1991) Sterol biosynthesis inhibitors: Their current status and modes of action. *Lipids*, **26,** 584-597.

Ragsdale, N.N. (1975) Specific effects of triarimol on sterol biosynthesis in *Ustilago maydis*. *Biochemica et Biophysica Acta*, **380,** 81-96.

Weete, J.D.; Sancholle, M.; Patterson, K.A.; Millar, K.S.; Huang, M.Q.; Campbell, F.; Van den Reek, M. (1991) Fatty acid metabolism in *Taphrina deformans* treated with sterol biosynthesis inhibitors. *Lipids*, **26,** 669-674.

MOLECULAR GENETIC ANALYSIS OF CARBOXIN RESISTANCE IN *USTILAGO MAYDIS*

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ABSTRACT

A gene which conferred resistance to the systemic fungicide carboxin was isolated from the maize smut pathogen, *Ustilago maydis,* using gene transfer techniques. The sequence of this gene showed a high degree of homology to succinate dehydrogenase iron-sulphur protein (Ip) subunit genes from a number of other organisms. Comparison of this sequence with that of an allele encoding the Ip subunit from a carboxin-sensitive *U. maydis* strain identified a two-base difference between the sequences. This divergence in the nucleotide sequences led to a single amino-acid change within the third cysteine-rich cluster of the Ip subunits, with a leucine residue being substituted for a histidine residue in the carboxin-resistant form. Site-directed mutagenesis and expression of the mutated gene in a carboxin-sensitive *U. maydis* strain showed that replacement of the histidine residue with leucine restored resistance to carboxin.

INTRODUCTION

Carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) is a systemic fungicide which is active against Basidiomycetes and is particularly effective in controlling bunt and smut diseases. Fungitoxic concentrations of carboxin inhibit respiration in *Ustilago maydis* by preventing the oxidation of succinate by succinate dehydrogenase (Mathre, 1971). This enzyme is composed of two subunits, a flavoprotein (Fp) and an iron-sulphur protein (Ip), which along with two other ubiquinone-binding proteins (CII-3 and CII-4) make up Complex II (succinate-ubiquinone reductase) of the respiratory electron transport chain in mitochondria (Figure 1). Although its precise mechanism of action is unclear, carboxin appears to prevent the transfer of electrons from succinate to ubiquinone by inhibiting reoxidation of the iron-redox centres of the Ip subunit (Ackrell *et al.*, 1977).

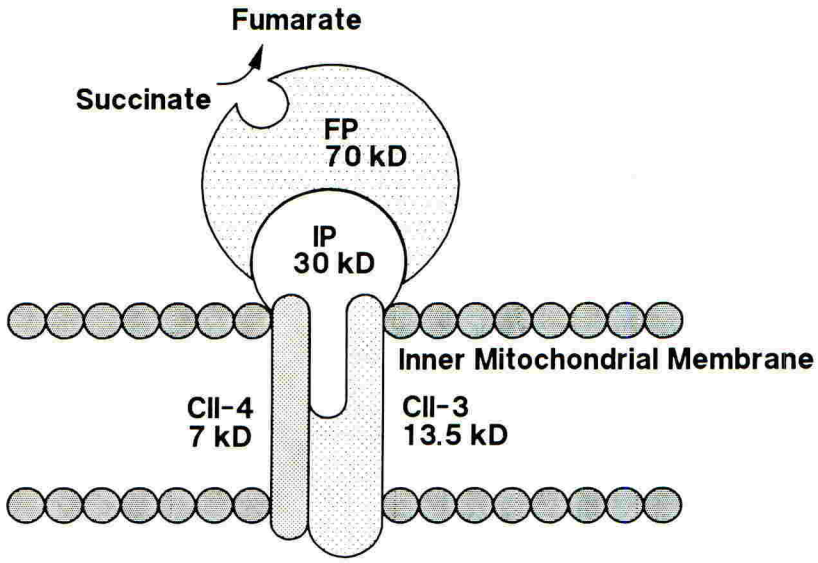

FIGURE 1. Schematic representation of the distribution of Complex II subunits.

Mutations at two nuclear loci lead to carboxin resistance in *U. maydis* (Georgopoulos *et al.*, 1972, 1975). The *oxr-1*B mutation, which confers a relatively high degree of resistance, has been shown to reduce the sensitivity of Complex II to carboxin (Georgopoulos *et al.*, 1972). This suggests that resistance in strains carrying this mutation is due to a conformational change at the site of action of carboxin.

Recent advances in the molecular genetics of *U. maydis* make it possible to routinely move DNA into and out of cells, and to gain expression of genes transferred into the cells (Tsukuda *et al.*, 1988; Wang *et al.*, 1988). Here we describe the application of modern fungal molecular genetic techniques to study the mode of action of, and the basis of resistance to, carboxin.

ISOLATION OF CARBOXIN RESISTANCE GENE

A genomic library from an *U. maydis* strain carrying the *oxr-1*B mutation was constructed by partially digesting genomic DNA with the restriction endonuclease *Sau*3A, to generated near-random DNA fragments (5-9 kb), and by ligating these into a unique *Bam*H1 site in plasmid pCM54 (Figure 2). This plasmid carries the *U. maydis hsp*70 promoter in transcriptional fusion with a gene conferring resistance to hygromycin B, an antibiotic which can be used as a selectable marker for gene transformation

in *U. maydis* (Wang *et al.*, 1988; Tsukuda *et al.*, 1988).

The plasmid gene library was transferred into a carboxin-sensitive *U. maydis* strain using standard PEG-mediated transformation procedures (Hargreaves & Turner, 1992). Transformants (*c.* 10,000) were recovered by selection on media containing hygromycin B (200 μg/ml) and then tested for their ability to grow on media containing concentrations of carboxin (2 μg/ml) that inhibited the growth of the sensitive strain. Five different carboxin-resistant transformants were recovered. Plasmids rescued from these transformants carried *U. maydis* DNA inserts which overlapped and transformed the *U. maydis* carboxin-sensitive strain to carboxin resistance at high frequency (*c.* 5,000 transformants/μg DNA). This indicated that the plasmids harboured identical DNA fragments containing the gene responsible for carboxin resistance.

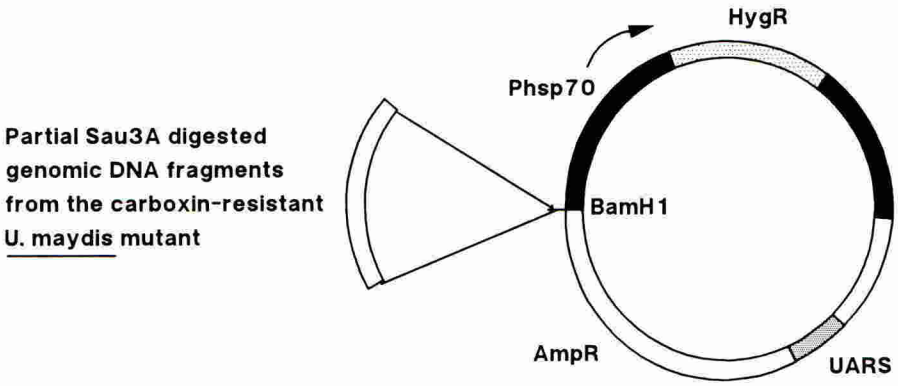

FIGURE 2. Construction of plasmid gene library from a carboxin-resistant mutant strain of *U. maydis* in *U. maydis* transformation plasmid, pCM54. Phsp70 - *U. maydis hsp*70 promoter, HygR - hygromycin B resistance gene, UARS - a *U. maydis* autonomously replicating sequence, AmpR - ampicillin resistance gene.

CHARACTERIZATION OF CARBOXIN RESISTANCE GENE

The gene was localised on the cloned DNA fragments by sub-cloning and deletion analysis and then sequenced (Keon *et al.*, 1991). A continuous open reading frame of 886 bases was detected which encoded a protein of *c.* 33,000 *k*D. Comparison of the deduced amino-acid sequence from this nucleotide sequence with EMBL and Genbank database accessions revealed extensive homology between the *U. maydis* carboxin resistance gene and the succinate dehydrogenase Ip subunit genes from humans, *E. coli* and yeast (66.3%, 58.1%, 64.3% identity respectively). The three cysteine-rich clusters at positions Ser105-Cys126 (Cluster 1), Glu198-Pro210 (Cluster 2) and Arg255-Pro267 (Cluster 3) were particularly well conserved (Figure 3).

The N-terminal amino-acid sequence of the *U. maydis* Ip subunit up to residue 50 was rich in basic (arginine) and hydroxylated (serine and threonine) amino-acids and part of this sequence formed a amphipathic helical structure. These features are characteristic of cleavable N-terminal presequences necessary for the import of mitochondrial proteins encoded by nuclear DNA (Hartl *et al.*, 1989).

```
MSLFNVSNGLRTALRPSVASSSRVAAFSTTAAARLATPTSDNVGSSGKPQ  50

HLKQFKIYRWNPDKPSEKPRLQSYTLDLNQTGPMVLDALIKIKNEIDPTL 100
      Cluster I
TFRRSCREGICGSCAMNIDGVNTLACLCRIDKQNDTKIYPLPHMYIVKDL 150
                                                Clust
VPDLTQFYKQYRSIEPFLKSNNTPSEGEHLQSPEERRRLDGLYECILCAC 200
er II
CSTSCPSYWWNQDEYLGPAVLMQAYRWMADSRDDFGEERRQKLENTFSLY 250
 Cluster III
RCLTIMNCSRTCPKNLNPGKAIAQIKKDMAVGAPKASERPIMASS       298
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FIGURE 3. Amino-acid sequence deduced from nucleotide sequence of *U. maydis* carboxin resistance gene. Positions of the three cysteine-rich clusters are bold and overlined.

NATURE OF MUTATION CONFERRING RESISTANCE TO CARBOXIN

The sequence of an allele encoding a carboxin-sensitive *U. maydis* Ip subunit was determined following PCR amplification of genomic DNA from a carboxin-sensitive *U. maydis* strain. Comparison of this sequence with that of the carboxin resistance gene revealed a two base difference between the sequences at nucleotide positions 758 and 759 (Broomfield & Hargreaves, 1992). This nucleotide divergence led to the substitution of a leucine residue for a histidine residue within the third cysteine-rich cluster of the carboxin-resistant Ip subunit (Figure 4). Confirmation that these nucleotide substitutions were responsible for carboxin resistance was obtained following site-directed mutagenesis of the sensitive Ip subunit gene so that it encoded a peptide identical to the resistant form. This *in vitro* mutated gene was transferred into a carboxin-sensitive strain of *U. maydis* and shown to restore resistance to carboxin.

DISCUSSION

Identification of the gene product responsible for conferring carboxin resistance in *U. maydis* as the succinate dehydrogenase Ip subunit, and the determination of the precise nature and location of the mutation leading to carboxin resistance within the resistant allele, allows a further assessment of the mechanism of action of carboxin to be made.

Carboxin-sensitive allele

```
750  CGA TGC CAC ACC ATC ATG AAC TGC 774
     Arg Cys His Thr Ile Met Asn Cys
```

Carboxin-resistant allele

```
750  CGA TGC CTT ACC ATC ATG AAC TGC 774
     Arg Cys Leu Thr Ile Met Asn Cys
```

FIGURE 4. Comparison of nucleotide sequences and deduced amino-acid sequences of *U. maydis* carboxin-sensitive and carboxin-resistant Ip subunits within the third cysteine-rich cluster. Codon CAC (position 757-760) in the carboxin-sensitive allele encodes for histidine, whereas codon (CTT) at this position in the carboxin-resistant form encodes for leucine.

Previous work had established that carboxin exerts its inhibitory effect by preventing electron transfer from the Ip subunit of succinate dehydrogenase to ubiquinone (Ackrell *et al.*, 1977). The third cysteine-rich cluster of the Ip subunit, in which the carboxin resistance mutation occurred, is associated with the S3 [3Fe-4S] high energy iron-redox centre. This centre is believed to be involved in the transfer of electrons to ubiquinone (Salerno, 1991) and carboxin is thought to act by preventing its reoxidation (Ackrell *et al.*, 1977). The results obtained in this study support the conclusion that carboxin interferes with the function of the S3 iron-redox centre of the Ip subunit. However, this interference with electron transfer to ubiquinone cannot be simply explained by carboxin binding to the Ip subunit. This is because the soluble form of succinate dehydrogenase (the flavoprotein subunit and the Ip subunit) is not affected by carboxin (Ulrich & Mathre, 1972) and photoaffinity labelling studies indicate that azidocarboxin does not selectively bind to the Ip subunit (Ramsay *et al.*, 1981). In simplest terms, carboxin could, therefore, be considered to exert its inhibitory effect by being lodged between the S3 centre of the Ip subunit and the ubiquinone binding site, in such a way as to hinder electron transfer to ubiquinone. Resistance to carboxin may, thus, be a result of a conformational change to the S3 iron-redox centre which allows electron transfer to proceed in the presence of carboxin.

REFERENCES

Ackrell, B.A.C., Kearney, E.B., Coles, C.J., Singer, T.P., Beinert, H., Yieh-Ping, W., Folkers, K. (1977) Kinetics of the reoxidation of succinate dehydrogenase. *Archives of Biochemistry and Biophysics*, **182,** 107-117.

Broomfield, P.L.E., Hargreaves, J.A. (1992) A single amino-acid change in the iron-sulphur protein subunit of succinate dehydrogenase confers resistance to carboxin in *Ustilago maydis*. *Current Genetics*, **21,** in press.

Georgopoulos, S.G., Alexandri, E., Chrysayi, M. (1972) Genetic evidence for the action of oxathiin and thiazole derivatives on the succinate dehydrogenase system of *Ustilago maydis* mitochondria. *Journal of Bacteriology*, **110,** 809-817.

Georgopoloulos, S.G., Chrysayi, M., White, G.A. (1975) Carboxin resistance in the haploid, the heterozygous diploid and the plant-parasitic dicaryotic phase of *Ustilago maydis*. *Pesticide Biochemistry and Physiology*, **5,** 543-551.

Hargreaves, J.A., Turner, G. (1992) Gene transformation in plant pathogenic fungi. In: *Molecular Plant Pathology: A Practical Approach, Vol. 1*, S.J. Gurr, M.J. McPherson and D.J. Bowles (Eds), Oxford, New York, Tokyo: IRL Press, pp. 79-97.

Hartl, F-U, Pfanner, N., Nicholson, D.W., Neupert, W. (1989) Mitochondrial protein import. *Biochemica et Biophysica Acta*, **988,** 1-45.

Keon, J.P.R., White, G.A., Hargreaves, J.A. (1991) Isolation, characterization and sequence of a gene conferring resistance to the systemic fungicide carboxin from the maize smut pathogen, *Ustilago maydis*. *Current Genetics*, **19,** 475-481.

Mathre, D.E. (1971) Mode of action of oxathiin systemic fungicides. III. Effect on mitochondrial activities. *Pesticide Biochemistry and Physiology*, **1,** 216-224.

Ramsay, R.R., Ackrell, B.A., Coles, C.J., Singer, T.P., White, G.A., Thorn, G.D. (1981) Reaction site of carboxanilides and of thenoyltrifluoroacetone in complex II. *Proceeding of the National Academy of Sciences, USA*, **78,** 825-828.

Salerno, J.C. (1991) Electron transfer in succinate:ubiquinone reductase and quinol:fumarate reductase. *Biochemical Society Transactions*, **19,** 599-605.

Tsukuda, T., Carleton, S., Fotheringham, S., Holloman, W.K. (1988) Isolation and characterization of an autonomously replicating sequence from *Ustilago maydis*. *Molecular and Cellular Biology*, **8,** 3703-3709.

Ulrich, J.T., Mathre, D.E. (1972) Mode of action of oyxathiin systemic fungicides. V. Effect on electron transport system of *Ustilago maydis* and *Saccharomyces cerevisiae*. *Journal of Bacteriology*, **10,** 628-632.

Wang, J., Holden, D.W., Leong, S.A. (1988) Gene transfer system for the phytopathogenic fungus *Ustilago maydis*. *Proceedings of the National Academy of Sciences, USA*, **85,** 865-869.

IDENTIFICATION OF MECHANISMS OF RESISTANCE IN LARVAE OF THE TOBACCO BUDWORM *HELIOTHIS VIRESCENS* FROM COTTON FIELD POPULATIONS

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ABSTRACT

Eggs and young larvae of *Heliothis virescens* were taken directly from cotton fields in various locations in the southern USA and examined for the presence of common mechanisms of resistance to cypermethrin. Nerve insensitivity was common in all the strains especially in the Saint Joseph, Louisiana strain in which over 50% of individuals were highly nerve insensitive. Delayed penetration of cypermethrin was found in all strains except that from Hondo, Texas. Metabolic resistance appeared rare. Some individuals of the Saint Joseph and Hondo strains were notable for high levels of metabolite production. Synergist studies gave little evidence for metabolic resistance although the use of discriminating doses with pbo suggested that metabolically resistant individuals were present in most strains.

INTRODUCTION

Resistance to the synthetic pyrethroids is common in the tobacco budworm *Heliothis virescens* throughout the cotton growing regions of the USA and in parts of central and south America. Continuous monitoring of resistance is conducted in the USA to provide data for effective management of the pest (Plapp *et al.*, 1990) although this information gives no indication of the mechanisms that are responsible for the resistance of these insects.

We have previously examined resistance mechanisms in strains of the related species *H. armigera* from Thailand (Ahmad & McCaffery, 1991) and India (West and McCaffery, 1992) and in a laboratory strain of *H. virescens* from the USA (Little *et al.*, 1989) and Colombia (Holloway and McCaffery, unpublished). Whilst metabolic mechanisms of resistance such as those conferred by enhanced monooxygenases or esterases are common in the former species they have been considered as rare or absent in field strains of *H. virescens* especially in the cotton-growing, southern states of the USA. Nevertheless, as indicated by a comprehensive program of adult vial assays, there has been a gradual increase in tolerance of the insects throughout the region over a number of seasons. Previous evidence suggests that the major mechanism of resistance to the synthetic pyrethroids in larvae of this species in the USA has been due to a form of nerve insensitivity (Plapp *et al.*, 1990) although the extent to which this has spread and the proportions of the populations that have acquired the mechanism have only been briefly examined (McCaffery *et al.*, 1991). There remains the possibility that the metabolic mechanism of resistance is being acquired by heavily selected strains of the insects and that this trend is occurring unnoticed. The work described here attempts to discover whether there is any basis to this by examining strains of insects from cotton fields in the USA in 1991 for the known major mechanisms of resistance.

MATERIALS AND METHODS

Insects

Samples of eggs or larvae of *Heliothis virescens* were collected from cotton plants in various locations in the US Cotton belt (Table 1) and shipped to Reading as eggs and young larvae. The insects were reared in a similar manner to that described previously (McCaffery *et al.* 1991). No insecticide selection was applied at any stage to any of these strains. All the experiments were

TABLE 1. Field strains of *Heliothis virescens* examined for presence of major resistance mechanisms

Strain name	Collection site	Date collected	Host plant
Tillar	Tillar, Arkansas	12 June 1991	Cotton
Hondo	Hondo, Texas	31 July 1991	Cotton
College Station	College Station, Texas	08 Aug. 1991	Cotton
Saint Joseph	Saint Joseph. Louisiana	25 Aug. 1991	Cotton
Leland	Leland, Mississippi	29 Sept.1991	Cotton

carried out using the field collected generation of the insects except for some synergist studies where the first laboratory generation was used.

Insecticides

Technical *cis*-cypermethrin (98.4%) and ^{14}C-*cis*-cypermethrin (2.0GBq.mMol^{-1}) was supplied by Shell Research Limited Sittingbourne, UK and ICI Agrochemicals, Jealott's Hill, UK. Piperonyl butoxide (2-(2-butoxyether)-ethyl-6-propyl piperonyl ether) (pbo) was supplied as technical (98%) material by Wellcome Environmental Health, Berkhamsted, UK.

Insecticide and Synergist Bioassays

Dose mortality studies on all the strains with serial dilutions of technical grade *cis*-cypermethrin in acetone were carried out (full data not shown). A discriminating dose of 0.1μg of *cis*-cypermethrin (LD_{99} of susceptible BRC strain) was used to distinguish between resistant and susceptible phenotypes. One μl drops of *cis*-cypermethrin in acetone were applied to the mesothorax of test insects (19 and 24 mg). Control insects were treated with acetone. Further larvae were treated on the mesothorax with a 1μl drop (20μg) of piperonyl butoxide in acetone. After 30 min these were treated with 0.1μg of *cis*-cypermethrin as described above. For each control and treatment group at least four replicates, each of ten insects were used. The insects were fed and mortality of both the synergised and unsynergised insects was assessed after 72 h as previously (McCaffery *et al.*, 1991). Synergist ratios were obtained using dose mortality assays (full data not shown).

Neurophysiological Assay

The effects of *cis*-cypermethrin on the spontaneous multiunit activity of nerves from larvae of each of the strains were measured at 25 ± 0.5°C using a modified 'cumulative dose response' assay (Gladwell *et al.*, 1990). *Cis*-cypermethrin in acetone was diluted directly into lepidopteran saline to give a final range of concentrations of 1.0 to 100nM (10^{-9} to 10^{-7}M). Third instar larvae were decapitated, opened dorso-medially and pinned out on a layer of Sylgard (Dow Corning). A peripheral nerve was picked up with an insulated stainless steel, suction electrode connected to a high gain, low noise amplifier and conditioning system (Neurolog, Digitimer Ltd.). Neural activity was monitored on an oscilloscope and recorded on magnetic tape. Nerve action potentials were identified by amplitude discrimination and a microcomputer used to record their frequency. The number of action potentials in each successive 15s period over a 5min control period in cypermethrin-free saline was recorded. The saline was then replaced with saline containing 1.0nM *cis*-cypermethrin, washed once and the recording continued. At 5min intervals the preparation was washed and immersed in fresh saline containing increasing concentrations of the insecticide. The end point of the assay was defined as the lowest concentration at which the frequency of action potentials was over five times greater than the mean value during the pre-treatment control period.

Assay of Penetration, Metabolism and Excretion

Individual fifth instar larvae, with a mean weight of 280±57mg (SEM±SD), in 50mm glass, carbowax-coated, petri dishes were topically dosed on the mesothorax with 1μl drops containing

0.05μg and 500Bq of ^{14}C-*cis*-cypermethrin. Insects were provided with small blocks of the standard artificial diet and left for 24h at 25°C. After 24h the the quantity of unpenetrated radioactivity, faecal radioactivity and the radioactive metabolite components of the faeces were determined all as described previously (Little *et al.*, 1989; Clarke *et al.*, 1990).

RESULTS

Toxicology and Synergism

A comparison of the resistance factors (full dose mortality data not shown) presented in Table 2 shows that, irrespective of location, resistance to cypermethrin increased markedly throughout the 1991 season. Values for the Tillar strain were very similar to those obtained with the susceptible BRC reference strain. The most resistant insects were found at St. Joseph and Leland whilst the Hondo and College Station strains were intermediate between these two extremes (Table 2). The mortality values obtained using the discriminating dose of 0.1μg very effectively ranked the the field strain insects in a similar manner with larvae of the Tillar strain being the most susceptible and larvae of the Saint Joseph and Leland strains being the most resistant (Table 2). Susceptible insects were present in all the field collections confirming their heterogeneous nature.

TABLE 2. Percentage mortality of third instar larvae of various strains of *Heliothis virescens* treated with *cis*-cypermethrin and the effects of pre-treatment with piperonyl butoxide.

Strain	Resistance Factor[a] at LD_{50}	Percentage mortality		Synergist Ratio[b] at LD_{50}
		0.1μg *cis*-cypermethrin	20μg pbo + 0.1ug *cis*-cyper.	
BRC	-	100	100	
Tillar	2.6	95	98	1.2
Hondo	14	45	70	2.6
College Station	30	45	75	1.8
Saint Joseph	103	28	40	2.2
Leland	97	30	55	4.1

[a] RF = Resistance Factor = LD_{50} Resistant Field Strain/LD_{50} of Susceptible BRC strain
[b] SR = Synergist Ratio = LD_{50} Insecticide alone/LD_{50} Insecticide + Synergist

Topical pre-treatment with PBO gave slight but non-significant synergism with cypermethrin in larvae of the field strains as indicated by the synergist ratios obtained in dose mortality bioassays (Table 2). In all the strains treatment with pbo and a discriminating dose of 0.1μg of *cis*-cypermethrin gave increased levels of mortality compared to that seen with cypermethrin alone (Table 2). The highly resistant Saint Joseph strain had the lowest mortality in the discriminating dose assay and pbo only marginally raised this mortality suggesting that enhanced monooxgenase activity was unlikely to be the major mechanism of resistance in this strain.

Neurophysiology

A range of phenotypes with respect to nerve insensitivity was seen in larvae of all the strains examined (Table 3). Every class of response was represented in the profile for each strain. However, there were clear indications of a substantial proportion of highly nerve insensitive larvae present in the St. Joseph and Leland strains. In the former strain the largest class of individuals in the profile was of the very highly resistant non-responding group. In both strains there was a trend towards a bi-modal distribution in responses with a minimum around 50nM cypermethrin. In contrast, the Tillar, Hondo and College Station strains had profiles in which the majority of larvae

TABLE 3. Phenotypic distribution of individual third instar larvae of susceptible strain (BRC) and field strains of *Heliothis virescens* showing responses in the cumulative dose response nerve assay.

cis-cypermethrin concentration (nM)	Number of individuals responding in each strain					
	BRC	Tillar	Hondo	College Station	Saint Joseph	Leland
1	23	13	9	10	4	11
5	7	5	8	6	6	3
10	0	2	8	3	4	5
50	0	3	3	4	1	1
100	0	2	3	5	3	2
>100	0	5	4	2	12	8
Total number of larvae tested	30	30	35	30	30	30

were susceptible although a few resistant individuals were identified. With the BRC susceptible strain the responses were confined to the two lowest concentrations (Table 3).

Penetration, Metabolism and Elimination

There were large differences between some of the strains in the degree of penetration of the applied dose are shown in Table 4. The Hondo strain was characterised by a large proportion of individuals with a very clearly defined high level of penetration of the applied dose of radiolabeled cypermethrin and this strain appeared very susceptible. The Leland strain was ranked as the next most susceptible in this respect whilst a large proportion of the Tillar, College Station and Saint Joseph larvae showed considerably lower levels of penetration (Table 4).

TABLE 4. Penetration of ^{14}C-*cis*-cypermethrin into fifth instar individuals of field strains of *Heliothis virescens*. The data are expressed as number of larvae from a sample of thirty showing various levels of penetration of the applied dose in 24h

Percentage penetration of applied dose	Number of larvae in each strain				
	Tillar	Hondo	College Station	Saint Joseph	Leland
81 - 100 %	3	18	1	3	3
61 - 80 %	6	10	9	11	15
41 - 60 %	13	1	13	10	10
21 - 40 %	8	1	6	6	2
0 - 20 %	0	0	1	0	0
Total number of larvae tested	30	30	30	30	30

The elimination profiles in Table 5 show a range of distributions of individuals with respect to their ability to void radiolabel from the body so that all the strains contained individuals in each class with respect to this parameter. The Tillar, Hondo and Saint Joseph strains all contained a high proportion of individuals able to eliminate a high proportion of the penetrated dose in 24h whereas somewhat fewer individual larvae of the College Station and Leland strains appeared to be able to do this. In general, there was little evidence for a marked ability to rapidly eliminate the penetrated toxicant but some larvae of the College Station and Hondo strains were particularly competent.

TABLE 5. Elimination of ^{14}C-*cis*-cypermethrin from fifth instar individuals of field strains of *Heliothis virescens*. The data are expressed as number of larvae from a sample of thirty showing various levels of elimination of the penetrated dose in 24h

Percentage elimination of penetrated dose	Number of larvae in each strain				
	Tillar	Hondo	College Station	Saint Joseph	Leland
0 - 20 %	6	7	6	3	2
21 - 40 %	5	4	7	7	7
41 - 60 %	8	5	9	6	13
61 - 80 %	9	11	4	8	6
81 - 100 %	2	3	4	6	2
Total number of larvae tested	30	30	30	30	30

The radiolabelled components of the faecal samples were analysed and the percentages of excreted material appearing as polar conjugates, primary metabolites and parent compound were calculated. Excretion of conjugates of primary metabolites was generally common but with all the strains examined there was little evidence of differences between strains. Nevertheless, there was a rather increased level of conjugate excretion from larvae of the St. Joseph strain compared with all the other strains. Slightly raised levels of acid production in the Tillar and Leland strains are unlikely to be significant. Evidence for significant monooxygenase action was not seen. There was little difference in the profiles of hydroxylated metabolite excretion between the strains although a few individuals of most of the strains, especially the Hondo strain, appeared to have a high activity in this regard and may represent individual resistant insects present at a lower frequency.

DISCUSSION

Heliothis species may possess physiological, biochemical and behavioural mechanisms of resistance (Nicholson and Miller, 1985). In this study we have attempted to define, using relatively rapid methodologies as far as possible, which of the mechanisms of delayed penetration, nerve insensitivity and enhanced monooxygenase activity, were present in strains taken directly from USA cotton fields.

Previous work (Gladwell *et al*., 1990; McCaffery *et al*., 1991) has shown the feasibility of distinguishing resistant and susceptible larvae on the basis of their response in a cumulative dose response assay performed on semi-isolated larval nervous systems. The assay gives a reliable indication of the frequency of appearance in a strain of individuals with particular levels of nerve insensitivity and is considered to give an efficient discrimination between phenotypes with respect to nerve insensitivity. The technique has been used to screen a large number of individuals to obtain a frequency profile for each strain. Such large scale testing with a quantitative electrophysiological technique has never been attempted before and we believe this gives a valuable insight into the occurrence of this mechanism. In the absence of a DNA probe diagnostic it is considered that this neurophysiological technique has very considerable utility. The St. Joseph strain contained a high proportion of highly nerve insensitive, resistant individuals. Other strains had lesser proportions of these resistant insects although they were present throughout all the strains and this confirms our view and that of other authors that this is the major mechanism or resistance to synthetic pyrethroids in *Heliothis virescens* in USA cotton.

From dose mortality assays there is no evidence of significant widespread synergism of cypermethrin with pbo in third instar larvae of any of the strains examined. Mild synergism of cypermethrin with pbo in larvae of the Leland strain suggests a possible minor role for monooxygen-

ases in these insects although the method cannot detect rare, strongly resistant individual insects. Using pbo with a discriminating dose of cypermethrin there was a small increase in larval mortality in all strains except Tillar suggesting that a small proportion of individuals of the Hondo, College Station, Saint Joseph and Leland strains were resistant to cypermethrin due to the presence of a pbo-suppressible monooxygenase. The mechanism, if present, occurs at a relatively low frequency.

In assessing the role of metabolism several factors were considered. First the quantity of penetrated radioactivity eliminated via the faeces was determined. This may include primary metabolites, conjugates of these as well as unchanged parent compound and it gives an overall indication of the ability of the insect to eliminate the toxicant. The Tillar Hondo and Saint Joseph strains contained rather more larvae capable of eliminating large quantities of the material than the College Station and Leland strains although the differences are marginal. The proportion of the excreted radioactivity represented by the various classes of metabolite was determined and whilst, in general, there were few differences between larvae of the different strains some strains contained individuals with better than average levels of elimination of radiolabeled metabolites. Conjugate production by some individuals of the Saint Joseph strain and monohydroxylated metabolite production by some individuals of the Hondo strain larvae were notable.

Whilst nerve insensitivity appears widespread the evidence for metabolic resistance is less convincing. There are clearly some individuals in the populations which are likely to be metabolically resistant and this is probably due to the presence of monooxygenases. Although the numbers appear small it needs to be borne in mind that for each strain we examined 30 individuals in detail. The finding that even a few of these individuals may have metabolic resistance is significant since these represent a large number in the field. Under increased selection pressure we would expect metabolic resistance to become significant in USA cotton.

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REFERENCES

Ahmad, M.; McCaffery, A.R. (1991) Elucidation of detoxication mechanisms involved in resistance to insecticides in the third instar larvae of a field-selected strain of *Helicoverpa armigera* with the use of synergists. *Pesticide Biochemistry and Physiology* **41**, 41-52.

Clarke, S.E.; Walker, C.H.; McCaffery, A.R. (1990) A comparison of the *in vitro* metabolism of *cis*-cyp ermethrin in a resistant and a susceptible strain of *Heliothis virescens*. *Proceedings 1990 Brighton Crop Protection Conference - Pests and Diseases*, 1201-1206.

Gladwell, R.T.; McCaffery, A.R.; Walker, C.H.(1990) Nerve insensitivity to cypermethrin in field and laboratory strains of *Heliothis virescens*. *Proceedings 1990 Beltwide Cotton Production Conferences*, 173-177.

Little, E.J.; McCaffery, A.R.; Walker, C.H.; Parker, T. (1989) Evidence for an enhanced metabolism of cypermethrin by a monooxygenase in a pyrethroid-resistant strain of the tobacco budworm (*Heliothis virescens* F.). *Pesticide Biochemistry and Physiology* **34**, 58-68.

McCaffery, A.R.; Gladwell, R.T.; El-Nayir, H.; Walker, C.H.; Perry, J.N.; Miles, M.M. (1991) Mechanisms of resistance to pyrethroids in laboratory and field strains of *Heliothis virescens*. *Southwestern Entomologist Suppl.* **15**, 143-158.

Nicholson, R.A.; Miller, T.A. (1985) Multifactorial resistance to transpermethrin in field collected strains of the tobacco budworm *Heliothis virescens* F. *Pesticide Science*, **16**, 561-570.

Plapp, F.W.Jr.; Campanhola, C.; Bagwell, R.D.; McCutchen, B.F. (1990) Management of pyrethroid resistant tobacco budworms on cotton in the United States. In: *Pesticide Resistance in Arthropods*, R.T. Roush and B.E. Tabashnik (Eds), New York: Chapman and Hall, pp. 237-260.

West, A.J.; McCaffery, A.R. (1992) Evidence of nerve insensitivity to cypermethrin from Indian strains of *Helicoverpa armigera*. *Proceedings 1992 Brighton Crop Protection Conference - Pests and Diseases*, In press.

EVIDENCE OF NERVE INSENSITIVITY TO CYPERMETHRIN FROM INDIAN STRAINS OF HELICOVERPA ARMIGERA

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ABSTRACT

Pupae of *Helicoverpa armigera* were collected from various cotton growing regions of India and transported to the Reading laboratory for rearing. Using a robust, tested and reliable electrophysiological assay and a wide range of concentrations of *cis*-cypermethrin large numbers of larvae of the first laboratory generation were tested for the presence of nerve insensitivity. The results showed that each strain had a unique response profile and for all the strains this differed from that of the susceptible strain. Some of the strains possessed individuals who demonstrated nerve insensitivity to a high degree.

INTRODUCTION

The cotton bollworm, *Helicoverpa armigera* (Hubner) is a polyphagous noctuid pest damaging a wide range of crops in many parts of the world including Asia, Africa and Australia. The synthetic pyrethroids are highly effective larvicides for this pest, being efficacious at low doses and maintaining favourable residual profiles on leaves. Combined with ready availability, generally low non-target toxicity and being relatively inexpensive they have been very widely used. However, the intensive use of these compounds has led to the development of resistance. The failure of synthetic pyrethroids to control *H. armigera* has been reported in Australia (Gunning *et al.*, 1984), Indonesia (McCaffery *et al.*, 1989), Thailand (Ahmad *et al.*, 1989; Ahmad & McCaffery, 1988) and India (McCaffery *et al.*, 1989; Dhingra *et al.*, 1988; Armes *et al.*, 1992).

Physiological and biochemical mechanisms of resistance to pyrethroids can be categorised into three types; delayed penetration, enhanced metabolism and nerve insensitivity. Of these nerve insensitivity has been considered to be the more important mechanism (Sawicki, 1985). It is generally agreed that it is the voltage-gated sodium channels that are the primary neuronal target sites for the pyrethroid insecticides (Nicholson & Miller, 1985) and that the compounds exert their effect by causing the open state of the gates of the sodium channel to persist. The observed neurophysiological effect of this is to induce repetitive firing in the peripheral and central nervous systems.

Evidence of a mechanism conferring resistance to both DDT and pyrethroids due to target site insensitivity has been known for some time. This nerve insensitivity to pyrethroids has been observed in lepidopteran species around the world and in *Heliothis virescens* (Gladwell *et al.*, 1990), and *H. armigera* (Gunning *et al.*,1984; Gunning *et al.*, 1991).

In this paper we report on investigations into the presence and degree of nerve insensitivity in field populations of *H.armigera* from India.

MATERIALS AND METHODS

Insects

Field collections of pupae were made in various locations in India between April and October 1992 and sent to the Reading laboratory via the Natural Resources Institute. Since no susceptible Indian strain was available we used the Sim Sim strain, derived from a susceptible Sudanese field strain and kept in the laboratory for several generations. All strains were bred and reared as described previously

(Ahmad & McCaffery, 1988) and all experiments were carried out at a constant temperature of 25±1°C.

Insecticide

Technical grade *cis*-cypermethrin (99%) was obtained from Shell Research Limited, Sittingbourne, UK and used to make up a 1mM stock solution in analytical grade acetone. The stock was then diluted with modified Weevers lepidopteran saline (Weevers, 1966) into thoroughly cleaned, Carbowaxed glassware to gave a range of concentrations from 0.0001nM to 100nM.

Neurophysiological assay

For each strain tested, third instar larvae weighing between 30 and 40mg were decapitated and a longitudinal dorso-median incision made, whereupon the insect was pinned onto a layer of Sylgard resin (Dow Corning) in a 50mm petri-dish. The intestinal tract, fat body and any loose tissue were removed and the preparation rinsed with several washes of saline, before being bathed in fresh saline. A peripheral nerve was picked up by means of a suction electrode constructed from 29 gauge stainless steel hypodermic tubing, the outer surface of which was coated with Epoxy resin. The preparation was grounded through a stainless steel entomological pin.

The recording electrode was connected to a high gain, low noise signal conditioning system, Neurolog (Digitimer Ltd., Herts, UK). The signal was displayed on an oscilloscope (Kenwood CS-8010) and an amplitude discriminator was used to distinguish the action potentials from the background noise. The nerve impulses were counted in epochs of 15s by a microcomputer.

The spontaneous multiunit activity (MUA) was recorded from the preparation whilst bathed in pesticide free saline. The saline was then aspirated and replaced with fresh saline containing 0.0001nM *cis*-cypermethrin, and the recording continued. After successive 5min periods the saline was aspirated off to be replaced by saline containing increasing concentrations of pesticide. The end point was determined by the lowest concentration of pesticide that caused a 5-fold increase in the MUA compared with the mean MUA of the saline control period.

Providing that the saline was replenished every five minutes the MUA remained relatively stable and the preparation would continue to be viable for over an hour. In order to confirm the stability of the preparation the MUA from several susceptible larvae was recorded for a period of 35 minutes before the saline was replaced with a 10 nM solution of *cis*-cypermethrin. In all cases application of the insecticide caused an increase in the MUA within five minutes showing that the preparations were capable of showing a response after forty minutes and that five minutes was a sufficient period of time for any response to manifest itself (Figure 1).

Around thirty larvae were used from each strain and to simplify comparisons between the strains the results from each strain are expressed in the form of a percentage of individuals responding at each of the concentrations.

RESULTS

Baseline MUA varied between preparations but did not bear any correlation with the degree of resistance of the larvae. Such variance is likely to be related to the variable number of nerves that are picked up by the suction electrode.

All the individual preparations from the Sim Sim susceptible strain clearly showed a distinct nerve intolerance to the pesticide demonstrated by a large increase in the MUA frequency, typically ten times the control value, upon application of the lowest or occasionally the second lowest concentration (Figure 2).

The responses from the field strain preparations (Figures 2-7) all differed from that of the known susceptible, although the majority of individuals again responded at either the first or second concentration. What we consider to be of more significance is the number of individuals responding at

the higher concentrations as this gives each of the strains a unique and characteristic profile (Figures 2-7).

Two of the strains Dhopibet and Pulladigunta, included a number of individuals in which the preparation did not respond even at the highest concentration, 100nM (maximum solubility of *cis*-cypermethrin in saline). These individuals were thus considered to have very highly insensitive nervous systems.

These two strains also showed a decrease in the proportion of individuals which responded at the first concentration, small in the case of the Dhopibet strain but clearly marked in the Pulladigunta strain and this leads to a well defined shift in the profile towards nerve insensitivity.

DISCUSSION

Most of the investigations concerned with pyrethroid pesticide resistance in Indian *H. armigera* have inclined to concentrate on the mechanism of enhanced metabolism (Phokela *et al*, 1989; Phokela & Mehrotra 1989; McCaffery *et al.*, 1989). Work carried out in this laboratory also concludes that enhanced metabolism, by mixed function oxidases, is a major mechanism of resistance in Indian strains of this insect. Such a conclusion is not unexpected when one considers the prominent polyphagous nature of the species and the mechanism is common in *H.armigera* for other areas of the world.

The results presented here clearly indicate that all of the field strains of *H. armigera* sampled from India included individuals with varying degrees of nerve insensitivity compared to the susceptible, and a number of insects exhibited this mechanism to a high degree.

Wilkinson (1991) postulated that even highly resistant larvae of *Heliothis virescens* that exhibit an elevated enhanced enzymic resistance mechanism may also possess a nervous system that has some tolerance to pyrethroids in order to provide sufficient time for metabolism to reduce the quantity of pyrethroid in the body of the insect to a non-lethal threshold. The initial concentrations used in the course of this study are extremely dilute (0.0001nM), but they are of the same order as was found to be present in the excised CNS of *H. virescens* following topical dosing with *cis*-cypermethrin (Wilkinson, 1991). Moreover, the fraction of sodium channels that need to be modified to cause repetitive discharging has been calculated, using tetramethrin on squid axons, (Lund & Narahashi, 1982) to be less than 0.1% or less, so that low concentrations of this order are legitimate.

Thus by utilising very low concentrations of pyrethroids and an extensive range of concentrations it is possible to distinguish, to a fine degree, the incidence and level of nerve tolerance in individuals of each strain. It is also feasible to chart the progress of an specific population with respect to nerve insensitivity over a series of seasons.

The results presented here suggest that there is presently a trend towards an increased incidence of highly nerve insensitive insects in various locations in India. Such populations currently exist in Australia (Gunning *et al.*, 1991), and in Thailand (Ahmad *et al.*, 1989). Since this resistance mechanism is more difficult to counteract from a management point of view, careful monitoring and sound pesticide management practices should be employed in order to prevent nerve insensitivity from becoming established in populations of the insect in India.

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REFERENCES

Ahmad, M.; Gladwell, R.T.; McCaffery A.R. (1989) Decreased nerve sensitivity is a mechanism of resistance in a pyrethroid resistant strain of *Heliothis armigera* from Thailand. *Pesticide Biochemistry and Physiology*, **35**, 165-171.

Ahmad, M; McCaffery A.R. (1988) Resistance to insecticides in a Thailand strain of *Heliothis armigera* (Hubner) (Lepidoptera: Noctuidae). *Journal of Economic Entomology*, **81**, 45-48

Armes, N.J.; Jadhav, D.R.; Bond, G.S.; King, A.B.S. (1992) Insecticide resistance in *Helicoverpa armigera* in South India. *Pesticide Science*, **34**, 355-364.

Dhingra, S.; Phokela, A.; Mehrotra, K.N. (1988) Cypermethrin resistance in the population of *Heliothis armigera* Hubner. *National Academy Science Letters*, **11**, 123-125.

Gammon, D.W. (1980) Pyrethroid resistance in a strain of *Spodoptera littoralis* is correlated with decreased sensitivity of the CNS *in vitro*. *Pesticide Biochemistry and Physiology*, **13**, 53-62.

Gladwell, R.T.; McCaffery A.R.; Walker, C. H. (1990) Nerve insensitivity to cypermethrin in field and laboratory strains of *Heliothis virescens*. *Proceedings 1990 Beltwide Cotton Production Conference*, 173-175.

Gunning, R.V.; Easton, C.S.; Balfe, M.E.; Ferris, I.G. (1991) Pyrethroid resistance mechanisms in Australian *Heliothis armigera*. *Pesticide Science*, **33**, 472-490.

Gunning, R.V.; Easton, C.S.; Greenup, L.R.; Edge, V.E. (1984) Pyrethroid resistance in *Heliothis armigera* (Hubner) (Lepidoptera: Noctuidae) in Australia. *Journal of Economic Entomology*, **77**, 1283-1287.

Lund, A.E.; Narahashi, T. (1982) Dose-dependent interaction of the pyrethroid isomers with sodium channels of squid axon membranes. *Neurotoxicology* **31**, 11-24.

McCaffery, A.R.; Ahmad, M. (1991) Elucidation of detoxication mechanisms involved in resistance to insecticides in the third instar larvae of a field selected strain of *helicoverpa armigera* with the use of synergists. *Pesticide Biochemistry and Physiology*, **41**, 41-52.

McCaffery, A.R.; King A.B.S.; Walker, A.J.; El-Nayir, H. (1989) Resistance to Synthetic Pyrethroids in the Bollworm, *Heliothis armigera* from Andhra Pradesh, India. *Pesticide Science*, **27**, 65-76.

McCaffery, A.R.; Walker, A.J.: Topper, C.J. (1991) Insecticide resistance in the bollworm, *Helicoverpa armigera* from Indonesia. *Pesticide Science*, **32**, 85-90.

Narahashi, T. (1985) Nerve membrane ionic channels as the primary target of pyrethroids. *Neurotoxicology* **6**, 3-22.

Nicholson, R.A.; Miller, T.A. (1985) Multifactorial resistance to transpermethrin in field collected strains of the tobacco budworm *Heliothis virescens* F. *Pesticide Science*, **16,** 561-570.

Phokela, A.; Dhingra, S.; Mehrotra, K.N. (1989) Pyrethroid resistance in *Heliothis armigera* Hubner I. Response to cypermethrin. *Proceedings of the National Academy of Science, India* **59b**, 373-381.

Phokela, A.; Mehrotra, K.N. (1989) Pyrethroid resistance in *Heliothis armigera* Hubner II. Permeability and Metabolism of Cypermethrin. *Proceedings of the National Academy of Science, India* **55b**, 235-238.

Sawicki, R. M. (1985) Resistance to pyrethroid insecticides in arthropods. In: *Insecticides*, D.H. Hutson and D.R. Roberts, (Eds), New York, Wiley, pp. 143-191.

Weevers, R.de G. (1966) A Lepidopteran saline: Effects of inorganic cation concentration on sensory,

Wilkinson, I.J. (1991) A pharmacokinetic investigation of the expression of pyrethroid resistance in third instar *Heliothis virescens* (Lepidoptera; Noctuidae) larvae. Ph.D Thesis, University of Reading.

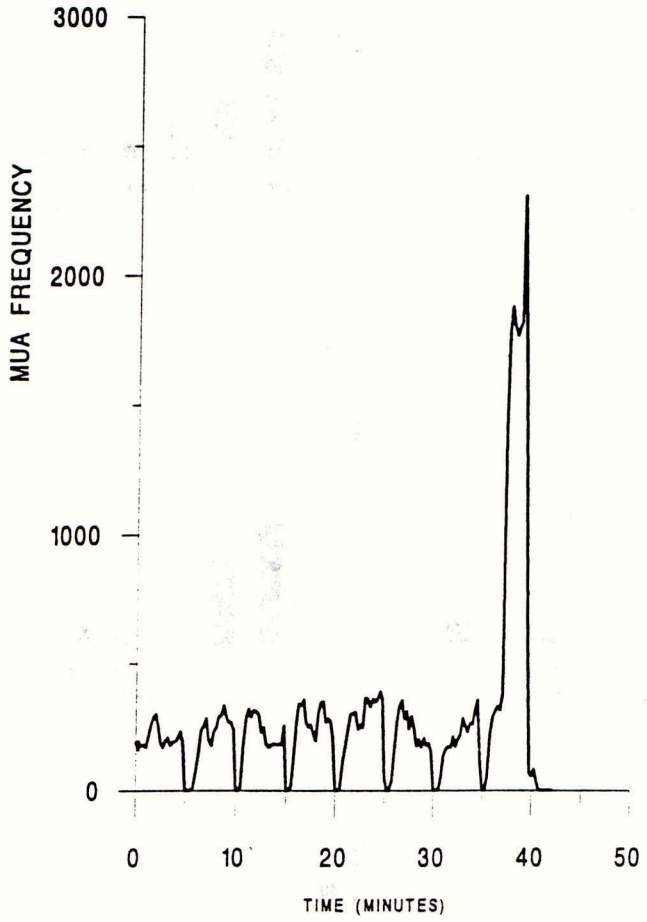
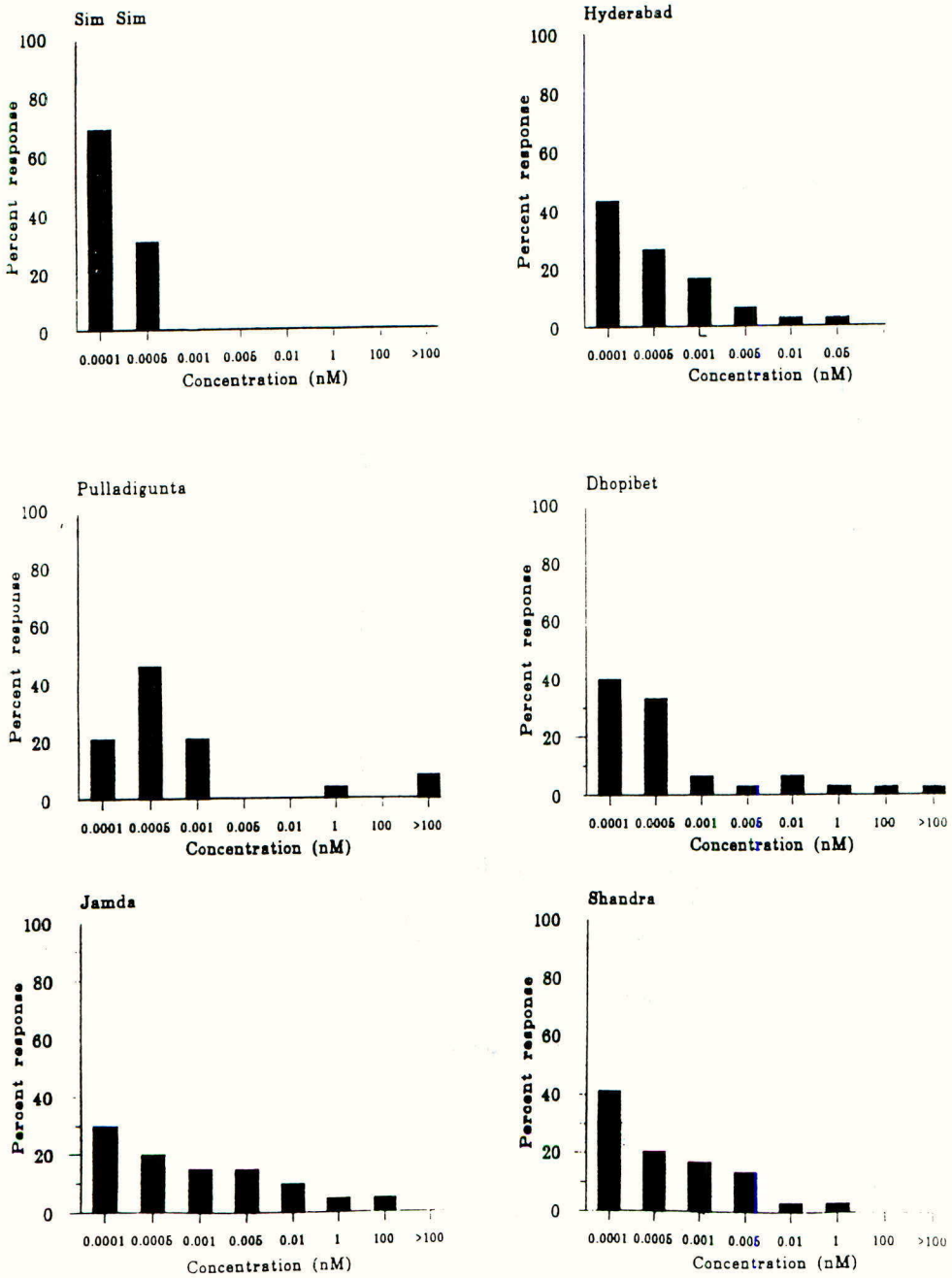


Figure 1. Graph showing impulse frequency from a third instar susceptible larva under pesticide free and pesticide containing saline.

Figures 2-7 Percentage of third instar larvae of various field strains of *Helicoverpa armigera* showing responses to various concentrations of *cis*-cypermethrin in the cumulative dose response neurophysiological assay.



PYRETHROID RESISTANCE IN THE POD BORER, *HELICOVERPA ARMIGERA*, IN SOUTHERN INDIA.

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ABSTRACT

Changes in the LD_{50} of *Helicoverpa armigera* to cypermethrin at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) and in the coastal cotton-growing region of Andhra Pradesh State during the period 1986-92 are summarised and discussed. A provisional discriminating dose of lug cypermethrin was evaluated at ICRISAT and changes in resistance to cypermethrin monitored throughout 1991/92. Resistance was related to seasonal changes in insecticide use; it was lowest in August and increased with progression of the season. Prospects for insecticide resistance management of *H. armigera* in Andhra Pradesh are briefly discussed.

INTRODUCTION

Synthetic pyrethroids were first used on cotton in S India in 1982 mainly against *Spodoptera litura* (F.) and *Earias* spp.; and were increasingly used against *Helicoverpa armigera* as it replaced these species. Pyrethroid resistance in *H armigera* was heralded in 1987 by widespread field control failures over much of the coastal cotton-growing belt of Andhra Pradesh (Dhingra *et al*, 1988; McCaffery *et al* 1989), and with a decline in average lint yields from over 430 to under 170 kg/ha (Anon, 1989 b and c). NRI, in collaboration with ICRISAT and Reading University, has been monitoring resistance in *H.armigera* since 1986. This paper summarises the techniques used, compares the data obtained in Andhra Pradesh, with particular reference to the 1991-92 season, and discusses future prospects for resistance management of *H.armigera* in southern India.

METHOD AND MATERIALS

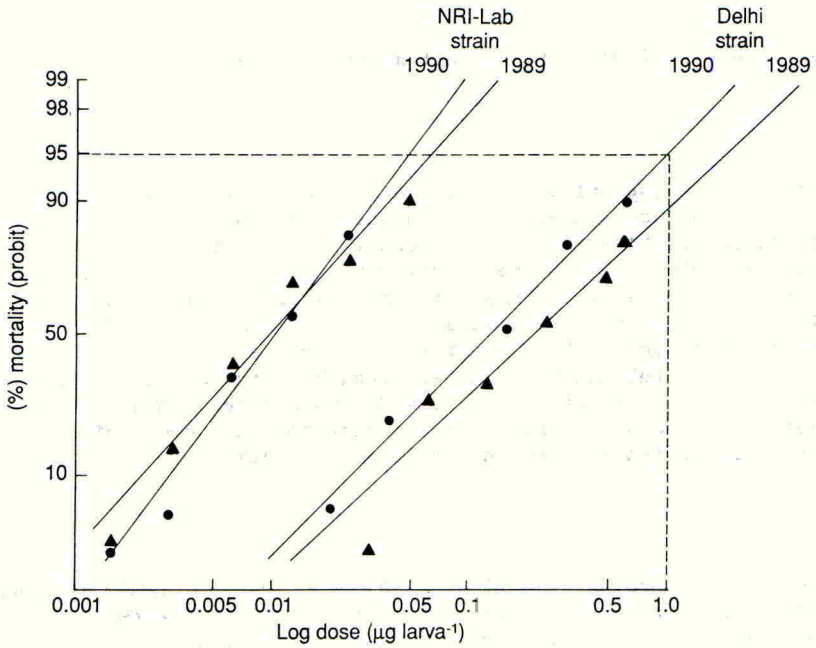
Dose/response monitoring

From 1986 to 1991 bioassays were performed on the F1 of larvae field-collected from sorghum, pigeonpea, chickpea and cotton at ICRISAT and farms in Andhra Pradesh. Cypermethrin (cis:trans, 1:1) (ICI Agrochemicals Ltd) was applied topically to larvae in the weight range 30-50 mg, as described by Armes *et al* (1992).

Discriminating dose monitoring

Two strains were used to calibrate a cypermethrin discriminating dose for *H.armigera* larvae weighing 30-50 mg. The NRI strain, originally from Sudan, was wholly susceptible; the other, from the Indian Agricultural Research Institute (IARI), Delhi, was slightly tolerant (Figure 1). A provisional discriminating dose of 1ug/larva, which killed 95% of the Delhi strain larvae, was derived (Figure 1).

FIGURE 1. Response to cypermethrin of the 'NRI laboratory' and 'Delhi' strains of *H.armigera*.



From June 1991 to April 1992, samples of 150-400 eggs and/or small larvae were taken from infested host plants every 1-2 weeks at ICRISAT, reared on diet to the 30-50 mg range and treated with the discriminating dose. Control larvae were treated with acetone alone. Larvae were held at 26±1°C and mortality assessed after 72 hours.

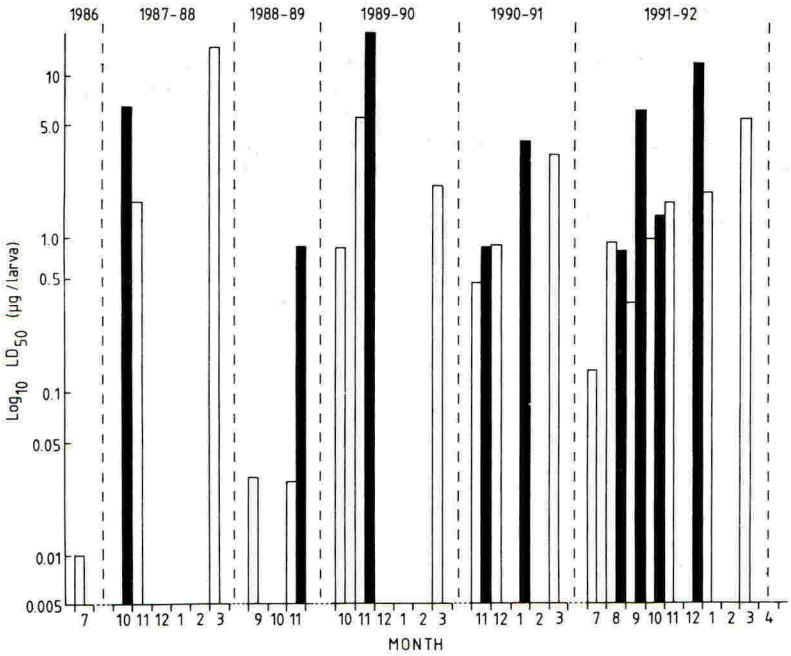
RESULTS AND DISCUSSION

Dose/response monitoring

Between 1986 and 1992 pyrethroid resistance varied considerably between and within years and locations (Figure 2) In July 1986, there was no evidence of tolerance to cypermethrin at ICRISAT, but by October 1987, field failures were reported from cotton in eastern A.P. and control problems were experienced at ICRISAT (Figure 2). In general, resistance levels over the 5 seasons increased as each season progressed. This was

particularly evident for the 1991/92 season for which there was continuous data.

FIGURE 2. Seasonal changes in cypermethrin resistance in *H.marmigera* from ICRISAT (unshaded) and Andhra Pradesh coastal cotton districts (shaded). Based on average monthly LD50 values; from McCaffery *et al.* (1989) and Armes *et al.* (1992).



In the cotton areas, increases in resistance could be attributed to local selection of resistant genotypes in response to applications of pyrethroids. However at ICRISAT, where pyrethroids were not extensively used, little local selection for resistance would have taken place. Seasonal increases in pyrethroid resistance at ICRISAT tended to reflect those recorded in the cotton areas in Eastern A.P., and may therefore have resulted from immigration of resistant moths into the Hyderabad area on the prevailing NE to E winds between October and December (Pedgley *et al.*, 1987; McCaffery *et al.*, 1989).

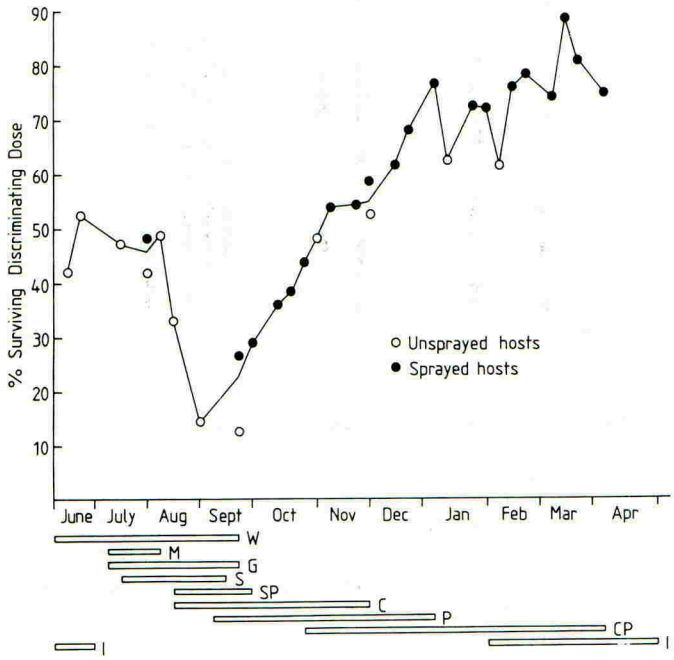
Discriminating dose monitoring

Ideally, monitoring should be able to detect resistant individuals at a phenotypic frequency close to 1%, a level not attainable with dosage/response assays (Roush and Miller, 1986). Moreover, the large numbers of insects required, inevitable time lag, and dubious accuracy of assays performed on the F1, are not compatible with a reactive IRM strategy.

Although a discriminating dose of 1ul was effective, the Delhi strain, on which it was based, was significantly more tolerant, and had a lower log-dose-probit (ldp) slope, than the NRI (Sudan) susceptible strain.

It has not been possible to isolate a homogenous, pyrethroid susceptible field strain in India, and, as a discriminating dose based on the NRI strain would overestimate pyrethroid resistance in Indian *H.armigera*, the Delhi strain was used as the standard. However, because of its low slope with cypermethrin, the ldp line of the Delhi strain significantly overlapped those of resistant field populations from southern India (Armes *et al.*, 1992). It was not therefore possible to accurately determine the frequency of resistance on the basis of a discriminating dose calibrated for this strain.

FIGURE 3. Changes in cypermethrin resistance in *H.armigera* at ICRISAT during 1991/92, based on percent larvae surviving a lug discriminating dose. Bars indicate periods of host-plant susceptibility to *H.armigera* at ICRISAT (W = weeds; M = mung bean; G = groundnut; SP = short duration pigeonpea; L = medium & long duration pigeonpea; C = cotton; CP = chickpea; I - irrigated vegetables).



At ICRISAT, resistance in the first *H.armigera* generation of the kharif (rainy) season in late June-late July 1991 was high, probably as a result of insecticide use during the March-May summer period on irrigated vegetables (Figure 3). Its subsequent decline could have resulted from early-season build up on unsprayed crops and weed hosts, and dilution by susceptible populations. However, by late October resistance had re-attained the early kharif level of about 45%. This increase was closely synchronised with the appearance of the first generation of moths from early planted cotton, which would have received 2-3 applications of insecticides. Resistance continued to increase until February, as an

overlapping succession of host crops were available (sorghum, pigeonpea, chickpea, groundnut), with the legumes receiving 1-6 sprays against *H.armigera*. Resistance remained high up to the end of the cropping season in early April (Figure 3).

Prospects for Insecticide Resistance Management in Andhra Pradesh

For a resistance management strategy to be successful it must be conducted on an area-wide basis, particularly for highly mobile pests like *H. armigera*. In Australia, a strategy where pyrethroid use is restricted to defined periods during the growing season, has been in operation since 1983 (Forrester and Cahill, 1987), and has effectively prevented field failures, despite steadily increasing resistance. Despite adherence to the strategy, pyrethroid resistance has continued to increase annually and field control has only been maintained with pyrethroid products because of tightly controlled rates and times of application. Factors contributing to the success of the Australian strategy, such as area-wide management, are consistent with farming conditions in developed countries.

In Andhra Pradesh area-wide management is likely to be extremely difficult in view of the large number of farmers involved and wide range of host crops grown at different times. Farmers are generally ill-informed as to the most appropriate management practices, application is often poor, tank mixes of different chemicals are frequent and the purity of some locally purchased chemicals has been questioned (Anon, 1990). Commonly, farmers do not scout their fields for eggs and only perceive *H.armigera* as a problem when larvae, or their damage, have become conspicuous. Control action is then less effective and selection for resistance more intense. It is hardly surprising therefore that resistance should appear under these conditions in a State which accounts for over 40% of pesticide sales in India (Anon, 1990) and where pyrethroids comprised 50-70% of all applications to cotton (Jayaswal, 1989).

In southern India, summer season (March-June) survival presents a potential weak link in the pest's life cycle. However, the increasing trend to grow host crops such as okra, eggplant and tomato under irrigation is almost certainly increasing survival over this period, as well as maintaining resistance when these crops are sprayed. Summer vegetables could well be an important contributory factor to the emergence of *H.armigera* as a major pest in cotton over the past ten years.

Clearly there is a need for IPM rationale, with room for major improvements in the efficiency of insecticide use. These would include need-based application, using thresholds for eggs and small larvae. A legislative, or incentive, system to restrict the use of pyrethroids on cotton, legumes and summer vegetabls during critical periods would also need to be implemented. In cotton, the potential and economics of varietal and agronomic changes to cotton crop management need also to be explored thoroughly.

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REFERENCES

Anon, 1989b. *Agricultural situation in India* **XLIII**, p801.

Anon, 1989c. *Agricultural situation in India* **XLIV**, p535.

Anon, 1990. Guidelines for the implementation of quality control of insecticides in Andhra Pradesh. *Pesticides Annual* 1989-90 **II**, 5-9.

Armes, N.J., Jadhav, D.R., Bond, G.S. and King, A.B.S. 1992. Insecticide resistance in *Helicoverpa armigera* in South India. *Pesticide Science* **34**, 355-364.

Dhingra, S., Phokela, A. and Mehrotra, K.N. 1988. Cypermethrin resistance in the populations of *Heliothis armigera* Hubner. *National Academy of Sciences Letters* **11** (4), 123-125.

Forrester, N.W. and Cahill, M. 1987. Management of insecticide resistance in *Heliothis armigera* (Hubner) in Australia. In: *Combating Resistance to Xenobiotics; Biological and Ecological Approaches*, M.B. Ford, D.W. Holloman, B.P.S. Khambay, and R.M. Sawicki, (Eds.), Ellis Horwood, UK, pp 127-137.

Jayaswal, A.P. 1989. Management of American bollworm on cotton in Andhra Pradesh. *Indian Farming*; July 1989, pp 6,7 & 17.

McCaffery, A.R., King, A.B.S., Walker, A.J. and El-Nayir, H. 1989. Resistance to synthetic pyrethroids in the bollworm, *Heliothis armigera* from Andhra Pradesh, India. *Pesticide Science* **27**, 65-76.

Pedgley, D.E., Tucker, M.R. and Pawar, C.S. 1987. Windborne migration of *Heliothis armigera* (Hubner) (Lepidoptera: Noctuidae) in India. *Insect Science and its Application* **8**, 599-604.

Roush, R.T. and Miller, G.L. 1986. Considerations for design of insecticide resistance monitoring programs. *Journal of Economic Entomology* **79**, 293-298.

PROBLEMS WITH ESTIMATING THE TOXICITY OF AMITRAZ TO SUSCEPTIBLE AND RESISTANT SPIDER MITES

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ABSTRACT

Acaricides, such as amitraz, that elicit behavioral effects pose special challenges to evaluating their toxicity because mites are inclined to abandon treated surfaces by moving onto untreated areas of bioassay apparatus. Thus, bioassay methodology may greatly influence the pest-pesticide interface for such compounds, altering both observed toxicity and expression of resistance. The influence of bioassay choice on estimates of toxicity of amitraz against susceptible *Tetranychus urticae* was investigated by comparing mortality observed in leaf disk, residual cell, and microimmersion bioassays. All three bioassay methods produced comparable and low estimates of the toxicity of amitraz to susceptible populations. Resistance to amitraz, readily isolated by selection with amitraz of a New York population of *T. urticae*, was manifest in residual cell and microimmersion (topical) bioassays as 15.5-fold and 6.4-fold increases in LC_{50}s, respectively. Selection reduced susceptibility to both amitraz and dicofol, indicating the presence of cross-resistance in this strain. We conclude that cross-resistance between dicofol and amitraz should be ascertained in programmes for managing mite pest populations but the bioassay method employed for assessing resistance must provide realistic estimates of responses to amitraz.

INTRODUCTION

Management of resistance in spider mites is of great concern to agriculturalists throughout the developed world. Success of resistance management hinges on using appropriate bioassays to detect differences in susceptibility of pests that are diagnostic of genetic changes, and on the identification of countermeasures, such as rotations of insecticides, predicated on known occurrences of cross-resistance. Indeed, recommendations formulated by the Insecticide Resistance Action Committee (IRAC), Fruit Crops Working Group (Leonard 1992) are based on classification of insecticides and acaricides into cross-resistance groups, to avoid repeated use of compounds that select common resistance mechanisms. Acaricides that elicit behavioral effects, such as feeding deterrence, increased locomotory activity, and repellency, pose particular challenges in this respect since mites are inclined to abandon treated leaf surfaces or, when enclosed on treated surfaces in cell bioassays, move to untreated areas of the enclosure (e.g., see Kolmes et al. 1991). With behaviorally-active acaricides, interactions between behavioral responses, innate toxicity of the chemicals, and bioassay-specific factors, such as the availability of untreated areas and mode of exposure of subjects to the chemicals, can influence greatly the outcome of toxicity estimates, as well as the expression of resistances.

Recent discrepancies in the literature regarding toxicity of amitraz to *Tetranychus urticae* Koch and cross-resistance between amitraz and dicofol exemplify well the difficulties experienced in bioassaying acaricides that strongly influence behavior. Fergusson-Kolmes et al. (1991) described the response to amitraz of near-isogenic dicofol-susceptible and -resistant strains of *T. urticae* and reported that the resistant one exhibited a pronounced cross-resistance to amitraz. Aveyard et al. (1992) challenged this report of cross-resistance, having failed to find reduced sensitivity to amitraz in 3 laboratory strains of *T. urticae* resistant to dicofol. Inspection of concentration-response data in these papers revealed very large differences in the toxicity of amitraz to susceptible mites, Aveyard et al. (1992) reporting comparable mortality from amitraz at concentrations 10-50 fold lower than that reported by Fergusson-Kolmes et al. (1991). Given the importance of eliminating this discrepancy, we examined the toxicity of amitraz using multiple bioassay methods, and we measured the responses of populations selected with amitraz or dicofol.

INFLUENCE OF BIOASSAY CHOICE ON ESTIMATES OF THE TOXICITY OF AMITRAZ

Methods

Studies were conducted at the Rothamsted Experimental Station, Harpenden, Herts, against the GSS strain, a susceptible laboratory population, obtained from Schering AG, Agrochemical Div., Berlin. The residual cell and microimmersion bioassays are described and contrasted in Farnham et al. (this volume) and Dennehy et al. (in press). The residual cell method confines groups of 20-25 young, adult, female mites within plastic (perspex) cells positioned over acaricide-treated leaves. Mites are placed within the cell after a leaf has been dipped in acaricide and allowed to dry; subjects are exposed to acaricide only by contact with the treated leaf. The microimmersion method is a newly-developed bioassay in which groups of 25 young, adult female mites are immersed in acaricide for 30 seconds, allowed to dry, and then treated subjects are confined on untreated leaves. The third method evaluated was a leaf disk bioassay described by Aveyard et al. (1992). With this residual contact method 10-15 young, adult, female mites were placed on 15 mm leaf disks that had been previously dipped in amitraz and allowed to dry. An important difference between the leaf disk method and the other two methods is that spider mites could walk off of the leaf disks onto the moistened, untreated substrate. 'Mitac 20', 200 g/l EC (Schering AG), and 0.01% Triton X-100 diluted in distilled water was used for acaricide solutions. Controls consisted of water and 0.01% Triton X-100. All assays were held 72 h post-treatment, under ambient light in a room maintained at 21 ± 2°C. Mites exhibiting repetitive (non-reflex) movement of more than one locomotory appendage after this period were recorded as alive. Concentration-response data were computed as mean mortality (±SEM), corrected for control mortality. Differences between bioassay methods were investigated using one-factor ANOVA (StatView, Brain Power Inc., Calabasis, California) of proportional mortality data transformed with the arcsin transformation.

Results and Discussion

Significant differences were found between mortality observed with amitraz in leaf disk, residual cell, and micrommersion bioassays ($P<.001$), but all methods yielded relatively low mortality of the susceptible strain (Figure 1). The three bioassay methods produced susceptibility estimates that were much closer to those reported by Fergusson-Kolmes et al. (1991) than to those reported by Aveyard et al. (1992). The leaf disk method produced the lowest mortality with amitraz. However, using this method, we

observed that up to 66 percent of test subjects abandoned the disks, this effect being most pronounced at the higher concentrations. We cannot explain fully the discrepancy between our results with this method and those of Aveyard et al. (1992), though we postulate that they scored as dead mites that had abandoned the leaf disks. Ignoring or scoring as dead individuals that move off treated surfaces of bioassays could exaggerate mortality estimates. However, this factor alone cannot account totally for the high mortality reported by Aveyard et al. (1992) at 100 ppm because we observed low repellency (only 12%) in bioassays of this concentration.

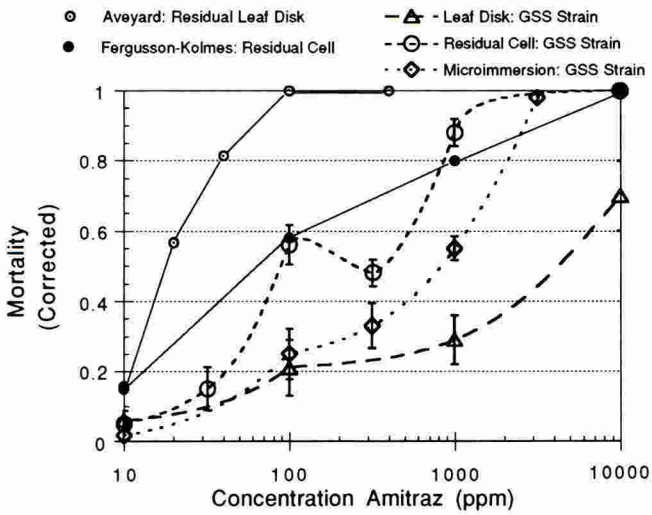

FIGURE 1. Influence of bioassay choice on estimates of the toxicity of amitraz to adult female *Tetranychus urticae*. Results of leaf disk, residual cell, and microimmersion bioassays are compared with toxicity estimates published in Aveyard et al. (1992) and Fergusson-Kolmes et al. (1991).

INFLUENCE OF BIOASSAY ON THE EXPRESSION OF RESISTANCE TO AMITRAZ IN ADULT *TETRANYCHUS URTICAE*.

Methods

Two populations were contrasted, the GSS strain, described above (non-selected), and the NYAmit1000 strain, an amitraz-selected strain of *T. urticae* from Cornell University. The NYAmit1000 strain originated from a highly characterized susceptible laboratory colony, Orchard-12, maintained since 1985 at Cornell University, Geneva, New York. Beginning in July, 1991, this strain was selected with increasingly higher concentrations of amitraz. Mites from the Orchard-12 strain were put into an isolated cage on plants that had been treated to run off with 10 ppm amitraz for the first four weeks, with 100 ppm for the next four weeks, and thereafter with 1000 ppm amitraz. By September, 1991, the population was growing well on plants sprayed with 1000 ppm amitraz and selection was sustained at this level throughout the period when bioassays were conducted. Mite responses to amitraz were plotted as mean (±SEM) mortality, corrected for control mortality, and analyses were conducted using the POLO probit analysis program (LeOra Software, Berkeley, California). Ninety-five percent fiducial limits for LC_{50} are presented. Owing to significant departures

from the probit model, differences between populations were analyzed using one-factor ANOVA as detailed above.

Results and Discussion

Resistance to amitraz was readily isolated from the New York population of *T. urticae* (Figure 2):significant differences were found between the GSS and the NYAmit1000 populations with both residual cell ($P<.001$) and microimmersion bioassays ($P<.001$). The LC_{50} of the NYAmit1000 population was 2300 ppm (1600-3100) and 3400 ppm (2300-4800), respectively, for residual cell and microimmersion bioassays. This contrasts with LC_{50}s for the susceptible GSS strain of 150 (100-220) and 530 (340-760) for residual cell and microimmersion bioassays, respectively (Figure 2). The low toxicity of amitraz necessitated using concentrations in excess of 1000 ppm in order to kill even low proportions of NYAmit1000 mites. All populations and methods yielded significant departures from a probit model. Ratios of LC_{50}s of the NYAmit1000/GSS populations were 15.5 in cell bioassays and 6.4 in microimmersion bioassays. Plateaus in response to amitraz were observed in residual cell bioassays of both populations (Figure 2). These plateaus were not observed in the microimmersion (topical) bioassays. We interpret this to be the result of behavioral responses of *T. urticae* to amitraz, expressed in residual bioassays. That is, over the concentrations involved in the plateau, increasing acaricide concentration enhanced avoidance of contact with the residue.

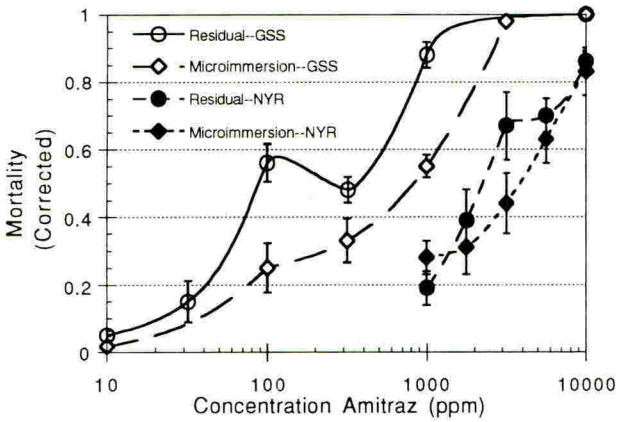

FIGURE 2. Influence of bioassay on the expression of resistance to amitraz in adult female *Tetranychus urticae*. A non-selected, susceptible population (GSS) and an amitraz-selected population (NYAmit1000) were tested in residual cell and microimmersion (topical) bioassays.

CROSS-RESISTANCE BETWEEN AMITRAZ AND DICOFOL IN A NEW YORK POPULATION OF *TETRANYCHUS URTICAE*.

Methods

Studies were conducted in 1991-2 at Cornell University, Geneva, New York. The Orchard-12 (susceptible) strain was split into two isolated populations, one maintained without selection and one selected with amitraz (NYAmit1000). In addition, the dicofol-selected strain, near-isogenic to

Orchard-12 (Fergusson-Kolmes et al. 1991), was maintained as a standard on caged plants that had been treated to run-off with 1000 ppm dicofol. After a total of 5 months of selection with amitraz, NYAmit1000 was bioassayed with 6-10 replications of residual cell bioassays of amitraz concentrations of 0, 10, 100, 1000, and 10,000 ppm. Similar assays were done of the Orchard-12 population and the dicofol-resistant strain. Results were computed for each population as mean ± SEM mortality observed for each concentration tested, corrected for control mortality. Differences between non-selected, amitraz-selected, and dicofol-selected populations were evaluated using one-factor ANOVA as noted above.

Results and Discussion

Five months of selection of the susceptible Orchard-12 population with amitraz resulted in significant reductions in susceptibility to both amitraz ($P<.001$) and dicofol ($P<.001$) (Figure 3), indicating the presence of cross-resistance between these acaricides in the population originating from New York. However, selection with amitraz reduced susceptibility to dicofol less than did selection with dicofol. This supports the conclusion of multiple factors, a single major gene plus modifiers, conferring dicofol resistance (Rizzieri et al. 1988) and indicates that cross-resistance between dicofol and amitraz involves a subset the factors. We hypothesize that the cross-resistance described herein involves the minor resistance factors described by Rizzieri et al. (1988), since the reduction in susceptibility to dicofol caused by selection with amitraz was not nearly as great as the reduction caused by selection with dicofol.

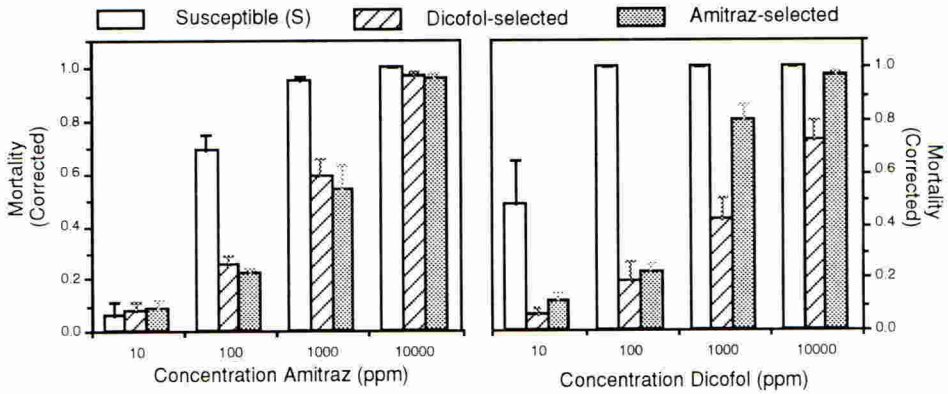

FIGURE 3. Cross-resistance between amitraz and dicofol is demonstrated by selection of a susceptible New York population of *Tetranychus urticae* with amitraz and testing susceptibility to amitraz and dicofol in residual bioassays. The dicofol-selected population, near-isogenic to the susceptible population, was used as a standard.

The toxicity of amitraz to susceptible forms of *T. urticae*, illustrated herein, is sufficiently low that relatively minor resistance mechanisms, such as those responsible for the above-mentioned 6- to 15-fold reduction in susceptibility to amitraz, rendered mites capable of surviving bioassays of very high concentrations, ones well in excess of common field rates. Our findings support the previous conclusion of Fergusson-Kolmes et al. (1991) regarding positive cross-resistance between dicofol and amitraz in mites from New York. Comparable reductions in susceptibility to amitraz were elicited by selection with dicofol or amitraz (Figure 3). We are

concerned about the ramifications that this cross resistance could have for selecting a mechanism of dicofol resistance. Resistance to dicofol is being successfully managed in some key systems (e.g., California cotton, Florida citrus, Brazilian citrus) by using rotations of materials that do not exhibit cross resistance. If this cross-resistance between dicofol and amitraz is expressed similarly in other pests populations, it could hamper efforts to sustain efficacy of both acaricides.

BIOASSAYING BEHAVIORALLY-ACTIVE ACARICIDES

We are unable to account fully for the the large discrepancy highlighted herein between previous reports of the toxicity of amitraz. However, we hypothesize that the problem centers around the effects of amitraz on spider mite behavior, effects that can result in mites abandoning treated surfaces in bioassays. Especially with behaviorally-active compounds, care must be taken when conducting bioassays to account for all subjects, and especially those that leave the pesticide treated area. Despite its low intrinsic toxicity to *T. urticae*, we are concerned not to undervalue the potential of amitraz as a mite control agent. Our results merely highlight the extreme difficulty of using laboratory bioassays to predict the likely field performance of behaviorally-active chemicals. Indeed, compounds that disrupt normal activity without imposing selective mortality offer tremendous scope for combating or avoiding resistance, and techniques for anticipating and documenting such effects under realistic conditions should be developed as a matter of priority.

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REFERENCES

Aveyard, C.S.; Richter, P.; Otto, D. (1992) Susceptibility of spider mite strains to amitraz and dicofol. In: Insecticides-Mechanisms of Action and Resistance, D. Otto and B. Weber (Eds), London, Intercept, pp 441-447.

Dennehy, T.J.; Farnham, A.W.; Denholm, I. (1992) The microimmersion bioassay: a novel method for the topical application of pesticides to spider mites (Acarina: Tetranychidae). *Pesticide Science*, (in press).

Fergusson-Kolmes, L.A.; Scott, J.G.; Dennehy, T.J. (1991) Dicofol resistance in *Tetranychus urticae* (Acari: Tetranychidae): cross-resistance and pharmacokinetics. *Journal of Economic Entomology*, 84,41-48.

Kolmes, S.A.; Dennehy, T.J.; Broadwater, E.B. (1991) Effects of residence time and webbing upon dicofol-avoidance behavior in two-spotted spider mites (Acari:Tetranychidae). *Experimental & Applied Acarology*, 12, 181-193.

Leonard, P.K. 1992. IRAC Fruit Crops Working Group: Spider mites. *In*, Resistance 91, Achievements and Developments in Combating Pesticide Resistance, I. Denholm, A.L. Devonshire and D. W. Hollomon (eds), Barking, UK, Elsevier. pp 41-47.

Rizzieri, D.A.; Dennehy, T.J.; Glover, T.J. (1988) Genetic analysis of dicofol resistance in two populations of twospotted spider mite (Acari: Tetranychidae) from New York apple orchards. *Journal of Economic Entomology*, 81, 1271-1276.

INSECTICIDAL ACTIVITY AND EXPRESSION OF PYRETHROID RESISTANCE IN ADULT *BEMISIA TABACI* USING A GLASS VIAL BIOASSAY.

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ABSTRACT

The reasons and methods for bioassaying *B. tabaci* are discussed and the published data reviewed. Large variations in baseline LC50's suggest that not all strains are truly susceptible. Screening small amounts of experimental material imposes constraints that preclude the use of the established leaf dip technique. An adult vial bioassay is presented that satisfies the requirements. Resistance to pyrethroids in the leaf dip bioassay is compared to that expressed in the vial test. The established method provides the greater discrimination.

INTRODUCTION

Within the last decade tobacco whitefly (*Bemisia tabaci*) has become a major pest of many crops in many countries. It is a pest because it causes direct feeding damage, exudes copious honeydew which is a substrate for fungi and creates harvesting difficulties (in cotton especially), and also transmits a large number of plant viruses. It is well established in glasshouse horticulture in continental Europe where it has severely disrupted the biological control programmes directed against *Trialeurodes vaporarorium* (Wilson & Anema, 1988). *B. tabaci* poses a constant threat to agriculture in the United Kingdom despite the efforts at quarantine.

Its elevation in status from an incidental to a primary pest has been attributed to many factors including increased use of insecticides and changes in cropping patterns (e.g. Dittrich *et al.*, 1986) as well as events that have lead to the dissemination of a biotype of *B. tabaci* that is highly fecund, has a wider host range, causes previously unreported physiological symptoms in host plants and in all examples tested to date is resistant to the major groups of insecticides (Costa & Brown, 1991; Cohen *et al.*, 1992).

These factors have increased the interest and urgency in conserving the existing xenobiotics for whitefly control and also stimulated the search for novel molecules to provide chemical control of this pest. It is therefore appropriate to review and assess the methods used to evaluate the laboratory efficacy of existing insecticides and to present a new technique.

THE ROLE OF BIOASSAYS

Bioassays fulfil a number of roles in arthropod toxicology. They may be used to screen for activity in the search for new chemistry, to test the laboratory efficacy of established and new insecticides, to provide information on the resistance and cross resistance patterns of a population and, by using synergists to assist in resistance mechanism studies. All of these roles may not be satisfied by a single technique.

The most widely reported bioassay method for *B. tabaci* is the adult leaf dip test. There are minor variations on this test but the principle is to expose adults of either one or both sexes to a leaf which has been dipped in formulated product. The leaf may be attached to a growing plant, in which case the adults are confined by a clip cage, or the leaf may be excised and kept moist and turgid on a bed of agar or moist filter paper in a small plastic cylinder.

TABLE 1. Log Dose Probit Mortality adult bioassay parameters reported for susceptible *Bemisia tabaci*. Test methods are 1= whole plant with leaf clip cage 2= leaf disc on agar gel 3= leaf with petiole in water 4= leaf dipped but not kept moist. F= females only tested, all others are mixed. RES signifies new Rothamsted leaf dip data.

REFERENCE	COMPOUND	LC50 (mg/l)	SLOPE	TEST METHOD	END PT (h)
Ahmed *et al.*, 1987	Endosulfan	1.1	1.18	3	24
Dittrich *et al.*, 1983	Endosulfan	1.6	3.40	2	24
Yassin *et al.*, 1989	Endosulfan	9.6	1.76	4	6
Prabhaker *et al.*, 1985	DDT	10.0	1.15	1	24
Dittrich *et al.*, 1983	DDT	11.5	2.40	2	24
Abdeldaffie *et al.*, 1987	Chlorfenvinphos	6.0	1.73	3	24
RES	Chlorpyriphos	2.8	4.70	2 F	48
Yassin *et al.*, 1989	Chlorpyriphos	17.4	1.56	4	6
Prabhaker *et al.*, 1985	Chlorpyriphos	190.0	2.14	1	24
Dittrich *et al.*, 1983	Dicrotophos	12.8	2.90	2	24
Ahmed *et al.*, 1987	Dimethoate	2.2	0.89	3	24
Dittrich *et al.*, 1983	Dimethoate	12.2	2.90	2	24
Prabhaker *et al.*, 1985	Fenthion	670.0	1.75	1	24
Prabhaker *et al.*, 1989	Malathion	127.0	4.00	1	24
Prabhaker *et al.*, 1985	Malathion	1440.0	1.69	1	24
Dittrich *et al.*, 1983	Monocrotophos	6.5	3.40	2	24
RES	Monocrotophos	24.8	4.90	2 F	48
Prabhaker *et al.*, 1989	Monocrotophos	80.0	3.80	1	24
Prabhaker *et al.*, 1985	Monocrotophos	820.0	3.17	1	24
Prabhaker *et al.*, 1985	Parathion	340.0	2.27	1	24
Prabhaker *et al.*, 1989	Parathion-methyl	67.0	4.50	1	24
Prabhaker *et al.*, 1985	Parathion-methyl	110.0	1.32	1	24
Horowitz *et al.*, 1988	Parathion-methyl	1900.0	2.43	2 F	24
Rowland *et al.*, 1991	Profenofos	0.7	2.00	2	48
RES	Profenofos	2.2	2.60	2 F	48
Dittrich *et al.*, 1983	Profenofos	4.9	4.40	2	24
Prabhaker *et al.*, 1989	Sulprophos	47.0	7.60	1	24
Prabhaker *et al.*, 1985	Sulprophos	80.0	1.70	1	24
Horowitz *et al.*, 1988	Sulprophos	300.0	1.90	2 F	24
RES	Bifenthrin	0.3	2.40	2 F	48
Rowland *et al.*, 1991	Cypermethrin	0.2	1.80	2	48
Dittrich *et al.*, 1983	Cypermethrin	2.9	1.70	2	24
RES	Cypermethrin	4.6	0.80	2 F	48
Horowitz *et al.*, 1988	Cypermethrin	75.0	1.22	2 F	24
Abdeldaffie *et al.*, 1987	Deltamethrin	0.1	0.77	3	24
Prabhaker *et al.*, 1985	Fenvalerate	20.0	1.23	1	24
Horowitz *et al.*, 1988	Permethrin	19.0	0.73	2 F	24
Prabhaker *et al.*, 1989	Permethrin	26.0	3.20	1	24
Prabhaker *et al.*, 1985	Permethrin	100.0	1.14	1	24

The LC50 values produced by the well established leaf dip method on ostensibly susceptible *B. tabaci* vary considerably between laboratories (Table 1). Cypermethrin LC50's differ by up to 375-fold while chlorpyriphos LC50's vary 68-fold. These differences may be the result of slightly different rearing, testing, holding and assessment methods, but the susceptibility of some of the strains must be questioned. The GH strain used as the susceptible reference by Prabhaker *et al.* (1985) was tested some time later (Prabhaker *et al.*, 1989) and in each retest produced lower LC50's and higher slopes. The susceptible strain used by Horowitz *et al.* (1988) was derived from the colony used by Prabhaker *et al.* (1985)

Resistance ratios are by definition dependent on the response of a susceptible strain. Although the delineation between susceptibility, tolerance and low level resistance to an insecticide is not always entirely clear, consistency of response over time is important. Homogeneity of a strain may be assumed by a high LDPM slope or verified by the use of biochemical/electrophoretic markers. Whatever the bioassay method the importance of a susceptible strain and associated baseline data can not be overestimated.

The leaf dip test is not a suitable bioassay method to screen experimental compounds. In our laboratories small amounts (<5ml) of dilute (<2%) technical material in acetone are provided by the chemists to the toxicologists. These compounds are expensive and time-consuming to produce and may be required to challenge a number of arthropod species.

To overcome these constraints a novel glass vial technique has been developed to test the contact activity of small amounts of technical compounds on adult *B. tabaci*. Preliminary tests with this technique to bioassay the parasitoids of *B. tabaci* (e.g. *Eretomocerus mundus* and *Encarsia lutea*) are also encouraging. A molecule toxic to *B. tabaci* and non-toxic to its natural enemies would provide a valuable tool for the management of this pest.

The impact of bioassay method on resistance expression has been widely reported (e.g. Dennehy *et al.*, 1983) and the development of a new bioassay method for testing adult whitefly provided the opportunity to investigate the expression of pyrethroid resistance in both the established leaf dip test and the new vial technique. An adult vial test has been used extensively in the USA for monitoring pyrethroid resistance in *Heliothis virescens* (Campanhola & Plapp, 1987) and a similar exercise is planned in the USA for *B. tabaci*.

DEVELOPMENT OF THE ADULT VIAL TEST

The optimum volume of liquid to give even coverage on the inside of the 10ml glass vial was determined using a red dye in acetone and 100µl found to be the most appropriate. New vials are used for all tests. The vials are soaked for 12h in Decon 75® to remove the shiny glass finish which causes the acetone to 'bead' and consequently leave untreated areas. The 100µl aliquot is dispensed into each vial which is then rolled on a purpose built roller for approximately 10min by which time the acetone evaporates. Adults are anaesthetised with CO_2 and 30 females are transferred to the vial where they are contained by a gauze lid held in place with a rubber band. Controls are handled in the same manner using vials coated with acetone. After exposure to the insecticide the adults

are transferred to a small (38mm diam. x 15mm high) plastic cylinder with an untreated leaf disc on a bed of agar gel. Mortality is scored at 24h and 48h with the latter considered the most appropriate end point. Control mortality of >15000 adults in 174 tests has averaged 7.5%.

The optimum exposure period was determined by exposing pyrethroid susceptible (SUD-S) female whiteflies to 0.1mg/l, 1mg/l 10mg/l or 100mg/l of technical cypermethrin in acetone for either 20min, 40min, 60min, or 120min and assessing at 48h (Figure 1).

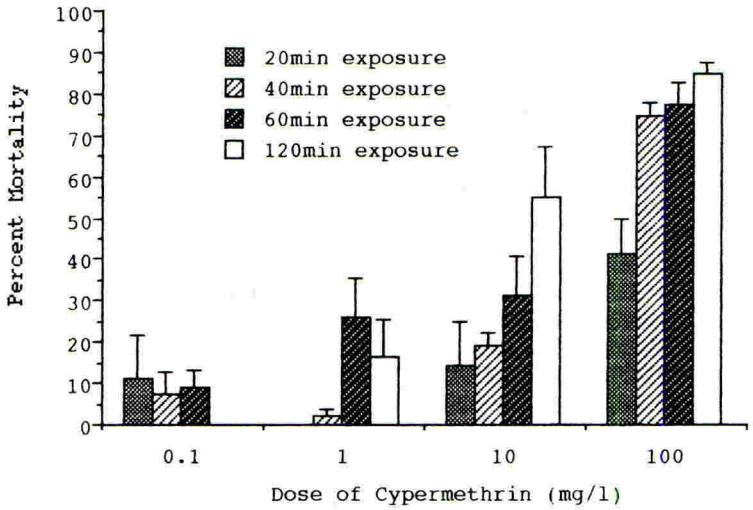

FIGURE 1. Mortality at 48h of pyrethroid susceptible *Bemisia tabaci* females exposed to cypermethrin in the glass vial test for 20, 40, 60, or 120min. All figures are corrected for control mortality. Bars indicate standard errors.

The 20min and 40min exposures gave the least consistent responses over the full dose range tested and the control mortality in the 120min exposure was higher (18%) than the 60min exposure (15%). For these reasons the 60min exposure was chosen for all subsequent tests.

COMPARISON BETWEEN THE ADULT VIAL TEST AND THE LEAF DIP TEST

A series of pyrethroids was tested on females of the SUD-S strain and a pyrethroid resistant strain (BELZ) using the vial test with 100µl of solution, 60min exposure and 48h end point (Table 2). Two of the pyrethroids were also tested with the leaf dip bioassay as reported in Rowland *et al.* (1991) using formulated product (Table 3).

The Log Dose - Probit Mortality (LDPM) determinations for bifenthrin and cypermethrin on susceptible *B. tabaci* with the vial test compare favourably with published data and with leaf dip data produced on the same strain at Rothamsted. However the resistance ratios derived for the BELZ strain differ considerably between the two test methods (Figure 2). The resistance ratios produced by the leaf dip bioassay are 3 to 4-fold higher than with the adult vial test (Tables 2,3)

TABLE 2. LC50's (mg/l), slopes and resistance ratios for females of two *Bemisia tabaci* strains tested using the adult vial technique.

Pyrethroid	SUDS		BELZ		RESIS.
	LC50 (limits)	Slope (se)	LC50 (limits)	Slope (se)	RATIO
Bifenthrin	0.3 (.26-.37)	2.8 (.25)	4.6 (3.2-7.8)	1.4 (.14)	15
Cypermethrin	9.5 (7.1-13)	1.6 (.13)	166 (105-265)	1.3 (.17)	17
Fenpropathrin	4.9 (2.-7)	2.6 (.50)	47.1 (32-67)	1.5 (.12)	10
Tefluthrin	1.0 (.7-1.2)	4.9 (1.1)	39.2 (23-56)	1.7 (.20)	39

TABLE 3. LC50's (mg/l), slopes and resistance ratios for females of two *Bemisia tabaci* strains tested using the leaf dip technique.

Pyrethroid	SUDS		BELZ		RESIS.
	LC50 (limits)	Slope (se)	LC50 (limits)	Slope (se)	RATIO
Bifenthrin	0.3 (.27-.44)	2.4 (.30)	21 (8.7-300)	0.9 (.12)	70
Cypermethrin	4.6 (1.9-40)	0.8 (.12)	248 (190-320)	2.0 (.18)	54

DISCUSSION

The influence of bioassay method on resistance ratios has been reported (Dennehy *et al.*, 1983). Roush & Miller (1986) suggested that bioassay techniques that are 'ecologically realistic' may improve resistance detection or at least indicate the extent of the field resistance problem. Recent experience with *B. tabaci* however does not confirm this. Rowland *et al.*(1991) reported that *B. tabaci* adults did not express resistance to cypermethrin in a set of field simulator experiments in spite of an 83-fold resistance ratio in the leaf dip bioassay.

The most appropriate method for detecting resistance is that method which provides greatest discrimination. The greatest discrimination between the pyrethroid susceptible and resistance strains of *B. tabaci* was produced using the leaf dip bioassay.

If the extreme LC50 values from Table 1 are disregarded on the basis of suspected resistance then the remaining values for each compound are similar enough to suggest that the leaf dip method is stable and robust. Resistance ratios should however be based on true susceptible strains tested in-house.

Sanderson & Roush (1992) found their glass vial bioassay technique inappropriate for testing *Trialeurodes vaporariorum* because the adults stuck to the residue inside the vial. We encountered this difficulty when high (>1000mg/l) concentrations were used which would preclude full LDPM line construction on highly resistant populations.

The glass vial technique described in this paper was designed with a specific objective i.e. to test the contact activity of small amounts of technical compounds on adult *B. tabaci* and whitefly parasitoids. The technique is now in routine use at Rothamsted and is providing valuable information on the activity of established and novel molecules.

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REFERENCES

Abdeldaffie, E.Y.A.; Elhag, E.A.; Bashir, N.H.H. (1987) Resistance in the cotton whitefly, *Bemisia tabaci* (Genn.), to insecticide recently introduced into Sudan Gezira. *Tropical Pest Management*, **33**, 283-286.

Ahmed, A.H.M.; Elhag, E.A.; Bashir, N.H.H. (1987) Insecticide resistance in the cotton whitefly (*Bemisia tabaci* Genn.) in the Sudan Gezira. *Tropical Pest Management*, **33**, 67-72.

Campanhola, C.; Plapp, F.W. Jr. (1989) Pyrethroid resistance in the tobacco budworm (Lepidoptera: Noctuidae): insecticide bioassays and field monitoring. *Journal of Economic Entomology*, **82**, 22-28.

Cohen, S.; Duffus, J.E.; Liu, H.Y. (1992) A new *Bemisia tabaci* biotype in the southwestern United States and its role in silverleaf of squash and transmission of lettuce infectious yellows virus. *Phytopathology*, **82**, 86-90.

Costa, H.S.; Brown, J.K.; (1991) Variation in biological characteristics and esterase patterns among populations of *Bemisia tabaci*, and the association of one population with silverleaf symptom induction. *Entomologia experimentalis et applicata*, **61**, 211-219.

Dennehy, T.J.; Granett, J.; Leigh, T.F.; (1983) Relevance of Slide-Dip and Residual Bioassay Comparisons to Detection of Resistance in Spider Mites. *Journal of Economic Entomology*. **76**, 1225-1230.

Dittrich, V.; Ernst, G.H. (1983) The resistance pattern in whiteflies of Sudanese cotton. *Mitteilungen der Deutschen Gesellschaft fur Allgemeine und Angewandte Entomologie*, **4**, 96-97.

Dittrich, V.; Hassan, S.O.; Ernst, G.H. (1986) Development of a new primary pest of cotton in the Sudan: *Bemisia tabaci*, the whitefly. *Agriculture Ecosystems and Environment*, **17**, 137-142.

Horowitz, A.R.; Toscano, N.C.; Youngman, R.R.; Georghiou, G.P. (1988) Synergism of insecticides with DEF in sweetpotato whitefly (Homoptera: Aleyrodidae). *Journal of Economic Entomology*, **81**, 110-114.

Prabhaker, N.; Coudriet, D.L.; Meyerdirk, D.E. (1985) Insecticide resistance in the sweetpotato whitefly, *Bemisia tabaci* (Homoptera: Aleyrodidae). *Journal of Economic Entomology*, **78**, 748-752.

Prabhaker, N.; Toscano, N.C.; Coudriet, D.L. (1989) Susceptibility of the immature and adult stages of the sweetpotato whitefly (Homoptera: Aleyrodidae) to selected insecticides. *Journal of Economic Entomology*, **82**, 983-988.

Roush, R.T; Miller, G.L. (1986) Considerations for Design of Insecticide Resistance Monitoring Programs. *Journal of Economic Entomology*, **79**, 293-298.

Rowland, M.; Hackett, B.; Stribley, M. (1991) Evaluation of insecticides in field-control simulators and standard laboratory bioassays against resistant and susceptible *Bemisia tabaci* (Homoptera: Aleyrodidae) from Sudan. *Bulletin of Entomological Research*, **81**, 189-199.

Sanderson, J.P.; Roush, R.T.; (1992) Monitoring Insecticide resistance in Greenhouse Whitefly (Homoptera: Aleyrodidae) with Yellow Sticky Cards. *Journal of Economic Entomology*, 85, 634-631.

Wilson, D.; Anema, B.P. (1988) Development of Buprofezin for control of *Trialeurodes vaporariorum* and *Bemisia tabaci* on glasshouse crops in the Netherlands and the UK. *Brighton Crop Protection Conference - Pests and Diseases 1988*, **1**, 175-180.

Yassin, K.M.; Bashir, N.H.H.; Gadalla, B.H. (1990) Effects of endosulfan, chlorpyrifos and their mixtures on *Bemisia tabaci* of Sudan Gezira. *Tropical Pest Management*, **36**, 230-233.

THE MICROIMMERSION BIOASSAY: A NOVEL METHOD FOR MEASURING ACARICIDAL ACTIVITY AND FOR CHARACTERISING PESTICIDE RESISTANCE IN SPIDER MITES

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ABSTRACT

A novel and versatile method is described for measuring the contact activity of acaricides against spider mites, and for detecting and characterising acaricide resistance. The microimmersion (MI) bioassay involves drawing batches of 25 mites into small pipette tips under vacuum pressure, immersing them for 30 s in 35ul of a test solution, and then confining the treated subjects on clean foliage in holding cells. Evaluations of amitraz, bifenthrin, chlorpyrifos and dicofol against susceptible strains of *Tetranychus urticae* showed the MI bioassay to be equally applicable to formulated and technical acaricides, and to give LC_{50} values that corresponded well with those from a conventional residue bioassay. The method also clearly diagnosed previously unreported resistance to bifenthrin in *T. urticae*, and a novel mechanism conferring strong, and apparently specific resistance to chlorpyrifos.

INTRODUCTION

Only two topical exposure bioassays have been published for spider mites (Helle and Overmeer, 1985): the slide-dip or slide-spray method (Voss, 1961), and a technique for topical application to individual mites (Harrison, 1961). The latter is considered too cumbersome for testing large numbers of subjects. Slide-based methods are well-established but encounter two major limitations. Firstly, they are only applicable for acaricides that act within the period (ca. 2 days) that mites can survive without feeding. Secondly, the inability to recover survivors is a major constraint on its use in acaricide resistance studies.

We describe here a novel topical-type bioassay that overcomes these limitations. This microimmersion (MI) bioassay is relatively fast and simple, allows recovery of treated subjects, and requires only small amounts of either formulated or technical acaricide solution. Using *Tetranychus urticae* as the test organism, we have evaluated this method for measuring the activity of four acaricides against a susceptible strain, and for diagnosing resistance to two of these chemicals.

DESCRIPTION OF THE MI BIOASSAY

The apparatus used to collect and treat mites requires four sections of plastic pipette tip as shown in Figure 1. 25 adult mites are collected under suction pressure into the small pipette tip, which fits over a filter paper disc on the end of Large Tip A. The latter (with the small tip still

attached) is then detached from the base piece and affixed to the first of two Gilson P100 pipettes. This pipette is held vertically and 35 ul of solution is drawn up slowly to fill the small tip and run a short distance into Large Tip A.

FIGURE 1. Configuration and dimensions of pipette tips used for MI bioassays.

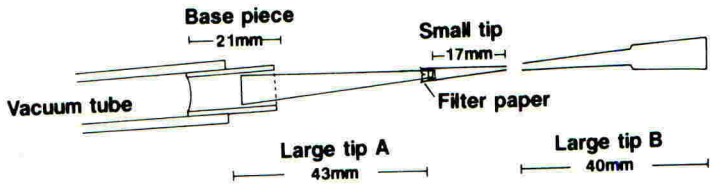
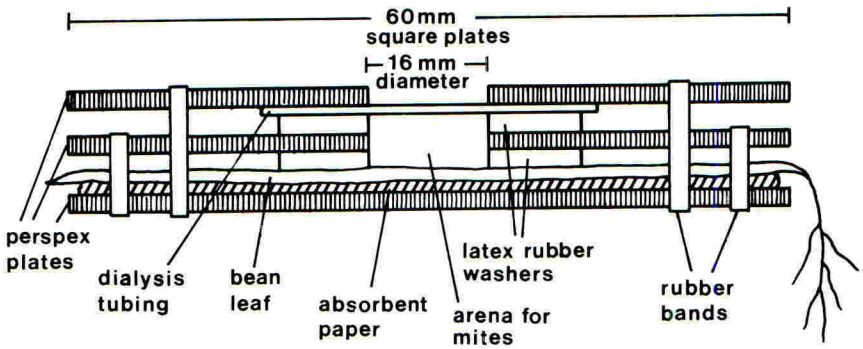


FIGURE 2. Construction of holding cell for confining mites.



Timing of the 30 s immersion period begins as soon as the fluid contacts the filter paper plug. After 20 s, the small tip containing immersed mites is detached from Large Tip A, inverted, and attached to Large Tip B (Figure 1) already in place on a second Gilson P100 pipette. At the end of the immersion period, the paper plug is removed with fine forceps and mites exhausted onto a filter paper disc. Great care is needed at this stage to avoid damaging the treated subjects (Dennehy *et al.*, in press).

After 10-20 s of drying on the filter paper, treated mites are transferred to holding cells (Figure 2) and enclosed within an arena formed on the upper leaf surface of an intact bean (*Phaseolus vulgaris*) seedling. Fully assembled cells are stored on racks for a prescribed period, and then disassembled to enable live and dead mites to be removed from the arena and counted. If required, survivors can be recovered and cultured on clean plant material.

EVALUATION OF THE MI BIOASSAY

Spider Mite Cultures

Two susceptible cultures, UKS and GSS, were obtained from Shell Research Limited, Sittingbourne, England, and Schering AG, Berlin, respectively. These gave very similar responses to the acaricides tested, and are referred to

collectively as the susceptible strain. NYR is a composite resistant population originating from intensively-sprayed apple orchards in New York State. Sub-cultures that had been maintained under strong selection pressure with bifenthrin or amitraz at Cornell University were bulked as a single strain. Susceptible and resistant strains were reared in separate rooms on 10-20 day old *P. vulgaris* seedlings at 23+3 °C with a 16 h photoperiod.

Acaricides and acaricide solutions

Diluted formulations of amitraz ('Mitac 20', 200 gl^{-1} EC, Schering AG), bifenthrin ('Talstar', 100 gl^{-1} EC, DowElanco Ltd), chlorpyrifos ('Dursban', 480 gl^{-1} EC, DowElanco Ltd) and dicofol ('Kelthane', 180 gl^{-1} EC, Rohm and Haas Ltd) were prepared as parts per million (ppm) of active ingredient on a weight-to-weight basis in distilled water containing 0.01% Triton X-100 as a surfactant.

Technical samples of amitraz (Schering Agrochemicals, Chesterford Park, England), bifenthrin (FMC Corporation, Princeton, New Jersey, USA) and chlorpyrifos (Promochem Ltd, St. Albans, England) were of 99.6%, >95% and 99.9% purity respectively. Solutions of technical products were initially prepared in acetone (AR grade) on a weight-to-volume basis. Immediately before immersion, these were diluted further with distilled water containing 0.0125% Triton X-100 to achieve final concentrations of 20% acetone, 0.01% surfactant, and the desired amount of acaricide. To compensate for the poor solubility of amitraz, vials containing serial dilutions of this chemical were held in an ultrasonic bath up to the period of immersion.

Design and scoring of bioassays

Tests involving 6-12 different concentrations of each chemical were replicated a minimum of 4 times and a maximum of 23 times to yield adequate data for statistical analysis. All bioassays were held for 72 h post-treatment, under ambient light in a room maintained at 21±2 °C. Mites exhibiting repetitive (non-reflex) movement of more than one locomotory appendage after this period were recorded as alive.

MI BIOASSAYS AGAINST SUSCEPTIBLE MITES

Responses to formulated bifenthrin, chlorpyrifos and dicofol were very homogeneous, and gave close fits to the probit model. LC_{50} values for bifenthrin and dicofol did not differ significantly, but both these compounds were more toxic at LC_{50} than amitraz, and less toxic than chlorpyrifos (Table 1). Hence, the ranking of toxicity in MI bioassays was chlorpyrifos > bifenthrin/dicofol > amitraz, with LC_{50} values ranging from 10 ppm for chlorpyrifos to 530 ppm for amitraz. Slopes of the probit lines also fell into three groups; those for chlorpyrifos (4.9) and dicofol (4.4) did not differ significantly, but were significantly different from that for bifenthrin (2.9). The slope for amitraz (1.5) was significantly lower than the other three.

Toxicities of the three technical acaricides evaluated with the MI bioassay followed the same pattern as that for the formulated products, with LC_{50} values ranked in the order chlorpyrifos > bifenthrin > amitraz (Table 1). The much larger standard error on the LC_{50} estimate for amitraz reflected a greater intrinsic heterogeneity in response, and problems with the solubility of this chemical at the concentrations required. As a result, the

slope of the probit line was again much lower for amitraz than for bifenthrin and chlorpyrifos. Difficulties with measuring the toxicity of amitraz in both bioassays are covered by Dennehy *et al.* elsewhere in these Proceedings.

At the LC_{50} level, technical bifenthrin and chlorpyrifos were 2.3-fold and 17-fold more toxic than the corresponding formulated products. There was no significant difference in this respect for amitraz. It is noteworthy, however, that the much lower slope obtained for technical (2.3) compared to formulated chlorpyrifos (4.9) resulted in very similar LC_{90} values (18 ppm and 21 ppm respectively) in the two bioassays. In contrast, slopes for technical and formulated bifenthrin did not differ significantly (Table 1).

COMPARISON OF MI AND RESIDUAL BIOASSAYS

Comparative data for formulated acaricides were obtained with a residual method employing holding cells identical to those used in the MI bioassay. In this case, leaves were dipped for 5 s in the required concentration of acaricide, and left to dry before being enclosed in the cells. 25 mites were then loaded into each cell, and mortality was again assessed after 72 h.

TABLE 1. LC_{50} values (ppm a.i.) and slopes of probit lines obtained with MI and residual bioassays

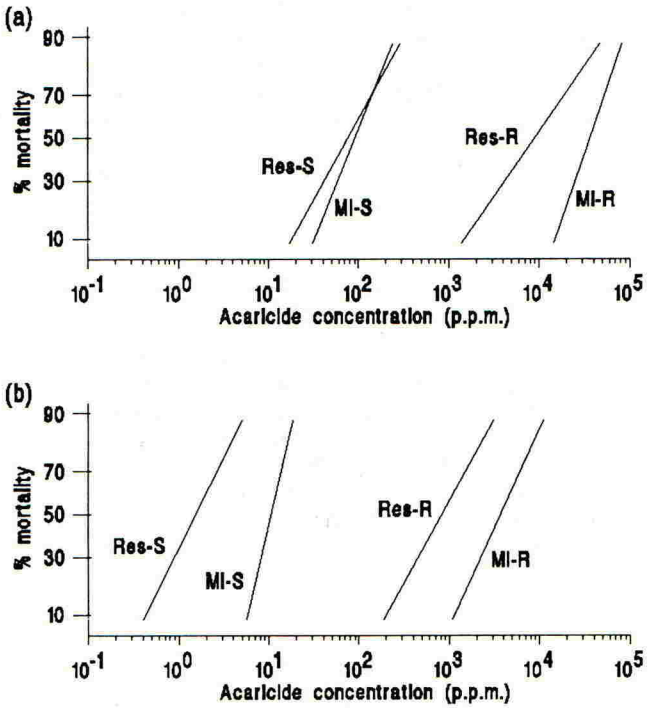
	Susceptible strain		Resistant Strain		
Chemical	LC_{50}[1] (x/÷ s.e.)	Slope[1]	LC_{50} (x/÷ s.e.)	Slope	R.F.[2]
(a) MI bioassays with formulated acaricides					
Amitraz	530[a] (1.20)	1.5[a]			
Bifenthrin	86[b] (1.08)	2.9[b]	36,000 (1.10)	3.4	420
Chlorpyrifos	10[c] (1.06)	4.9[c]	3,500 (1.11)	2.6	350
Dicofol	61[b] (1.06)	4.4[c]			
(b) MI bioassays with technical acaricides					
Amitraz	480[a] (1.41)	1.1[a]			
Bifenthrin	37[b] (1.09)	3.3[b]	7,200 (1.04)	3.4	190
Chlorpyrifos	5.8[c] (1.13)	2.3[c]	2,100 (1.03)	11.0	360
(c) Residual bioassays with formulated acaricides					
Amitraz	150[a] (1.21)	1.2[a]			
Bifenthrin	71[b] (1.15)	2.1[b]	8,200 (1.20)	1.7	120
Chlorpyrifos	1.4[c] (1.16)	2.3[b]	760 (1.13)	2.1	540
Dicofol	75[ab] (1.07)	3.3[c]			

[1]For each type of bioassay, superscripts identify LC_{50} values and slopes that do not differ significantly at the 95% probability level.
[2]Resistance factor at LC_{50} relative to the susceptible strain.

The relative toxicity of the four compounds was very similar in residual and MI tests (Table 1). LC_{50} values were ranked in an identical manner, and differed less than 10-fold between bioassay methods for all four chemicals. At LC_{50}, amitraz and chlorpyrifos were 3.5-fold and 7.1-fold more toxic respectively in the residual bioassay, whereas differences for bifenthrin and dicofol were small and not significant. The most notable difference between bioassay methods was that probit lines were consistently

and steeper under the more uniform exposure conditions of the MI bioassay.

FIGURE 1. Probit lines for the susceptible (S) and NYR (R) strains tested with commercial formulations of (a) bifenthrin and (b) chlorpyrifos. MI = microimmersion bioassay; Res = Residual bioassay.



DIAGNOSIS OF RESISTANCE TO BIFENTHRIN AND CHLORPYRIFOS

The MI bioassay also proved extremely effective in diagnosing two very potent and previously unreported types of resistance present at high frequencies in the NYR strain. In the case of bifenthrin (Figure 3a), with which the strain had been intensively selected in the laboratory prior to testing, the resistance factor was 3.5-fold higher than with the residual bioassay, even though the two methods gave very similar results for the susceptible strain. In addition, the MI probit line for NYR was twice as steep as that obtained with the residual bioassay (Table 1). Resistance factors for chlorpyrifos were more consistent between bioassays, since the difference in toxicity recorded for susceptible mites was also apparent in bioassays against the NYR strain (Figure 3b). Since even the susceptible strain used for this work was highly tolerant of related chemicals such as parathion and azinphosmethyl, this resistance appears distinct to that conferred by older and more widely-distributed organophosphate (OP) resistance mechanisms. Cross-resistance between bifenthrin and chlorpyrifos is unlikely, as we have recorded equally high resistance to chlorpyrifos in a strain of *Panonychus ulmi* retaining full susceptibility to bifenthrin.

Bifenthrin is becoming widely-used as an acaricide in several cropping systems, and there have, to our knowledge, been no prior reports of

resistance in Tetranychid mites. Despite its history of laboratory selection with bifenthrin, results for the NYR strain highlight a potential for strong resistance in field populations of *T. urticae*, and the need to establish its occurrence and cross-resistance characteristics.

The discovery of novel and seemingly very specific resistance to chlorpyrifos in NYR questions the validity of regarding OPs as a single cross-resisted group in an acaricide rotation strategy (Leonard, 1992). Indeed, it is likely that in many areas chlorpyrifos can still play an important role in managing otherwise OP-resistant populations. As with bifenthrin, however, the key requirement is to determine the current incidence of chlorpyrifos resistance and to resolve its genetic and biochemical basis. This work is currently underway at Rothamsted and Cornell University, exploiting the MI bioassay as a precise diagnostic and research tool.

CONCLUSIONS

The MI bioassay appears to satisfy all the requirements of a novel and much-needed topical-type bioassay procedure for spider mites. Its applicability to technical as well as formulated products overcomes a major constraint of foliar bioassays, and the requirement for only 35 ul of solution per batch of 25 mites may offer major advantages for screening the efficacy of newly-synthesised or experimental compounds. For research on acaricide resistance, the method shows considerable promise as a means of diagnosing resistance genotypes and phenotypes, of selecting resistant populations, and for applying precise quantities of materials for radiolabel and synergism studies to elucidate resistance mechanisms.

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REFERENCES

Dennehy, T.J.; Farnham, A.W.; Denholm, I. The microimmersion bioassay: a novel method for the topical application of pesticides to spider mites. *Pesticide Science* (in press).

Harrison, R.A. (1961) Topical application of insecticide solutions to mites and small insects. *New Zealand Journal of Science*, 4, 534-539.

Helle, W.; Overmeer, W.P.J. (1985) Toxicological test methods. In: *Spider Mites: Their Biology, Natural Enemies and Control*, W. Helle and M.W. Sabelis (Eds), Amsterdam: Elsevier, pp. 391-395.

Leonard, P.K. (1992) IRAC Fuit Crops Working Group spider mite resistance management strategy. In: *Resistance 91: Achievements and Developments in Combating Pesticide Resistance*, I. Denholm, A.L. Devonshire and D.W. Hollomon (Eds), Barking, UK: Elsevier, pp. 41-47.

Voss, G. (1961) Ein neues Akarizid-Austestvarfahren fur Spinnmilben. *Anz. Schaedlingskd.* 34, 76-77.

TESTING INSECTICIDE USE STRATEGIES: A MODEL GRAIN STORE SYSTEM FOR THE SAW-TOOTHED GRAIN BEETLE, *ORYZAEPHILUS SURINAMENSIS*

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ABSTRACT

An experimental grain store model is described for the study of the ecology and population genetics of insecticide resistance in the saw-toothed grain beetle, *Oryzaephilus surinamensis*. Efficacy of control is shown to be related to the extent of insecticide treatment. The model demonstrates the ability of refuges to act as foci of infestation, and examines their role as a source of susceptible beetles that may retard the evolution of resistance.

INTRODUCTION

Insecticides remain indispensable in many integrated management strategies for the control of insect pests. There is an increasing requirement for improvements in the use of insecticides so that while control of target species is maintained, collateral impact on the environment is reduced, and the evolution of resistance is discouraged. Effective strategies must take account of the ecology, life-history and population genetics of pest species. Many analytical and simulation models have demonstrated the potential of these and other operational factors to affect the population dynamics and spread of resistance in local pest populations. Of primary importance are the relative fitnesses of genotypes, the strength of insecticide dose, the presence of refuges (wherein individuals escape selection by insecticide), and migration (Georghiou & Taylor, 1977; Caprio & Tabashnik, 1992).

A small number of laboratory studies have confirmed the importance of migration and the degree of dominance of the resistance allele (Taylor *et al.*, 1983). Indirect evidence of the influence of selection and migration on local, regional and worldwide patterns of resistance exists for several insect pest species (Unruh, 1990; Raymond *et al.*, 1991).

There is a lack of experimental studies that directly test the different insecticide use strategies derived from theoretical studies (but see Curtis & Rawlings, 1980). Some theoretical models have argued that refuges are an inevitable and fortuitous feature of pest population structure and insecticide treatment methods, and they are often regarded as a source of susceptible individuals that may retard the spread of resistance. Yet, no studies have demonstrated the influence of refuges in pest populations.

The comparatively simple post-harvest ecosystem of the grain store is prone to infestation by a range of pest species. Control is achieved through the practices of general hygiene, cooling and drying of grain, and the mainly prophylactic application of insecticides. Insecticide treatments are employed in 53.4% of farm and 85.4% of commercial grains stores in England and Wales. Pirimiphos-methyl, the most widely used of the 4 grain protectant compounds available in the UK, is used in 39.6% of farm and 69.4% of commercial grain stores (Prickett *et al.*, 1990).

One of the most widespread insect pest of stored grain in the UK, and the model species for this study, is the saw-toothed grain beetle, *Oryzaephilus surinamensis*. It has been detected in 4.8% of farm and 14% of commercial grain stores in England and Wales. Respectively, 27% and 82% of strains from these sites were found to be resistant in the laboratory to a discriminating dose of pirimiphos-methyl (Prickett *et al.*, 1990).

This paper describes an experimental model system that mimics the conditions in a grain store containing refuges. A compromise is struck between the conflicting requirements of reproducing the biological and operational conditions of the pest in the grain store, and restricting the number of variables to permit a meaningful interpretation of results. It is used to study the population genetics and ecology of *O. surinamensis* under putative chemical control strategies. Results of partial fabric treatments are compared and the role of refuges in maintaining insect pest populations is considered.

MATERIALS AND METHODS

Blockboard bins, measuring 2 x 2 x 1.25 m, were set up indoors on plywood bases. The interior was lined with renewable kraft paper, taking care to seal any gaps and overlapping edges to prevent insects escaping. A 10 cm-wide aluminium foil strip was glued around the interior at a height of 1 m and coated with Fluon (ICI Advanced Materials, UK), an aqueous suspension of polytetrafluoroethylene. This limited vertical movement and prevented escape.

Refuges of thin card were glued to the bin walls to form a pocket 20 cm long and 8 cm high. Each bin contained a total of 16 refuges, 4 to a wall, arranged in 2 rows (at heights of 0.315 and 0.685 m). Refuges were positioned at 0.225 m and 0.123 m (upper row) and at 0.775 m and 0.177 m (lower row) along from the far left of each wall. A standard volume of kibbled, pesticide-free, winter wheat cv. Avalon (weighing approximately 120 g) was placed in each refuge.

Actellic-40 wettable powder (40% wt/wt pirimiphos-methyl; ICI Plant Protection Division, UK) in water was applied to run off from a hand-held sprayer operated from within the bin, and allowed to dry overnight. An average dose of 20 mg/m^2 pirimiphos-methyl was applied to either 1, 2 adjacent, 3 or 4 walls of 4 bins. A control bin was left unsprayed. To assess the concentration delivered and the persistence of the residue over time, 8 x 8 cm squares of kraft paper were pinned to the walls before spraying. These were removed and quantitatively analysed for pirimiphos-methyl residues at bi-monthly intervals throughout the experiment (Chamberlain, 1990).

The laboratory strain 7022/1 of *O. surinamensis* was used because it comprised approximately 4% of pirimiphos-methyl-resistant phenotypes, as determined by a standard discriminating dose bioassay of 156 mg/m^2 of the insecticide (Prickett *et al.*, 1990) (96.0% knockdown, n = 571). It was a relatively new culture to the laboratory (c. 3 years), collected from a UK commercial grain store, and so was assumed to possess more of its original genetic variability than longer established strains. An initial population of 2000 beetles, drawn from different aged laboratory cultures, was divided so that 125 beetles were counted into each of 16 glass tubes containing 20 g kibbled wheat. The day after the bins had been treated, single tubes and their contents were slid into the kibbled wheat in each refuge in an attempt to reduce the initial agitation dispersal. The bins were covered with plywood sheets to ensure the near-dark conditions typical of grain stores. Throughout

the experiment temperature varied between 18 and 27 °C, and relative humidity varied with the ambient conditions. Both were recorded on a thermohygrograph.

Pesticide-free Avalon wheat was fumigated overnight with methyl bromide to ensure that it did not contain live insects. The wheat was allowed to stand for 3 days so that any remaining methyl bromide vapour would diffuse out. On the 10th day 65 kg were placed in the centre of the floor of each bin.

The duration of the experiment was determined by the rate at which changes in population size and phenotypic proportions would have become apparent. Three pitfall-type PC traps (Cogan *et al.*, 1990), containing a small amount of laboratory culture food to sustain trapped beetles until their retrieval, were placed beneath the surface of the grain at week 11. They were removed at week 13, after which time sufficient live beetles were present in even the most extensively treated bins to conduct resistance phenotype determination tests. Live and dead beetles were counted, and the resistance phenotypes of survivors were determined with discriminating dose bioassays. The experiment was completed after 16 weeks (≤ 2 generations), when the differences in trap totals between bins indicated the relative success of each treatment. The refuges were emptied and the live and dead insects counted. Resistance phenotypes were determined where possible.

RESULTS

Numbers of beetles recovered are presented in Table 1. Live beetles were recovered from all bins. More live beetles than in the initial populations were recovered from the control and single treated wall bins. It is not possible to infer whether there was an overall increase in numbers in the other bins as estimates of trap efficiency were not available. Fabric treatments reduced populations by at least an order of magnitude, even in the single treated wall bin. They produced a 16-fold or greater decrease in live beetles trapped in the grain compared with the control bin, although there was no clear relationship between the number of live beetles recovered after 16 weeks (l) and the number of treated walls (t) in the treated bins (regression: $l = 481 - 83.4\ t$, $P = 0.356$; $r^2 = 0.12$). Numbers of beetles found dead in the traps varied inversely with the number of walls treated ($l = 64.5 - 17.1\ t$, $P = 0.057$, $r^2 = 0.833$).

Increasing the number of treated walls reduced the mean number of live beetles recovered over a range of 4 orders of magnitude in refuges on treated walls. There was a weak inverse relationship between the number of treated walls and the number of live beetles recovered from refuges on treated walls ($l = 53.0 - 12.2\ t$, $r^2 = 0.15$, $P = 0.008$). By comparison, there was a 1 order of magnitude reduction in the mean number of live insects in refuges on untreated walls. There was a strong inverse relationship between the treatment and live beetles recovered ($l = 256.4 - 77.9\ t$; $r^2 = 0.53$, $P \approx 0.000$). Refuges on untreated walls harboured more beetles than those on treated walls in the same bin (two independent sample t-test: bin with 1 treated wall, $t_{13\ d.f.} = 2.52$, $P = 0.026$; 2 treated walls, $t_{10\ d.f.} = 5.01$, $P = 0.0005$; 3 treated walls, $t_{3\ d.f.} = 4.90$, $P = 0.016$).

Discriminating dose tests detected no selection for resistance in any of the bins when compared with phenotypic proportions in the original cultures (Table 2). Only beetles from refuges in the single treated wall bin exhibited a significant change (contingency test, $\chi^2_{1\ d.f.} = 23.80$, $P < 0.001$), and this was towards an increase in the proportion of susceptibles. Comparisons of

TABLE 1. Numbers of 7022/1 *O.surinamensis* recovered from grain trap samples (after 13 weeks) and refuges on pirimiphos-methyl-treated (initially 20.4 mg/m²) and untreated walls (after 16 weeks).

	Grain		Refuges on treated walls		Refuges on untreated walls	
Number of walls treated	Alive	Dead	Alive	Dead	Alive	Dead
0	>7800	700	-	-	247.9[a] (19.9)	19.8 (1.8)
1	277	50	130.3 (10.0)	441.8 (98.7)	201.7 (26.6)	15.8 (3.8)
2	485	32	15.3 (5.9)	191.7 (19.6)	82.7 (12.1)	17.6 (7.8)
3	251	2	2.6 (0.7)	68.3 (8.4)	23.8 (4.3)	38.3 (23.8)
4	77	3	0.3 (0.1)	82.3 (9.1)	-	-

[a] Mean values with standard errors in brackets

TABLE 2. Percentage knockdown (kd) of 7022/1 *O. surinamensis* with 156 mg/m² discriminating dose of pirimiphos-methyl.

Number of walls treated	Grain (11-13 weeks) kd	n	χ^2	Refuges (16 weeks) kd	n	χ^2
0	94.1	271	1.46 n.s. [a]	-	-	-
1	97.8	223	1.50 n.s.	99.0	1886	23.80 ***
2	94.7	360	0.80 n.s.	97.8	617	3.04 n.s.
3	94.7	228	0.59 n.s.	94.7	111	0.43 n.s.
4	100.0	63	2.63 n.s.	-	0	-

[a] Contingency $\chi^2_{1\ d.f.}$ values derived from comparison of totals of 23 live and 548 dead beetles (96.0% kd) in tests on original cultures.

knockdown of beetles from grain in the treated bins with that from the control bin indicated no selection for resistance. Beetles from the single treated wall bin were significantly more susceptible ($\chi^2_{1\ d.f.} = 4.03$, $P < 0.05$). There was a small increase in the proportion of resistant phenotypes in the refuges as the extent of the treatment increased, consistent with weak selection for resistance. The differences in proportions were significant overall ($\chi^2_{2\ d.f.} = 17.12$, $P < 0.005$), with only that of the pair-wise comparison between the bins with 2 and 3 treated walls barely failing to achieve significance ($\chi^2_{1\ d.f.} = 3.464$, $P > 0.05$). Beetles from refuges in the bin with

2 treated walls had a higher knockdown than those from the grain ($\chi^2_{1\ d.f.} = 6.31$, $P < 0.05$). There was no significant difference in the bins with 1 treated wall ($\chi^2_{1\ d.f.} = 2.70$, $P > 0.05$) and with 3 treated walls ($\chi^2_{1\ d.f.} = 0.003$, $P > 0.99$).

Insecticide residues decayed from a mean concentration at application of 20.4 mg/m^2, through 7.25 mg/m^2 after 8 weeks, to 2.89 mg/m^2 at the end of the experiment (16 weeks).

DISCUSSION

The concentration of insecticide applied was considerably lower than a recommended field dose (equivalent to 400 mg/m^2, Muggleton *et al.*, 1986), and decayed by an order of magnitude during the experiment. Nevertheless, it was sufficient to cause heavy mortality. Yet the discriminating dose tests revealed no increase in the proportion of resistant phenotypes in beetles sampled from the grain and only a small increase in those from the refuges from treated bins. It is likely that resistance alleles are present at negligible frequencies in this strain (subsequent dose-response tests indicate a resistance factor of 1.78 at the LD50 in the 7022/1 strain relative to the Laboratory Susceptible strain), so almost all individuals possessing them will be heterozygous at any resistance locus. The relationship between the response to long-term exposure to waning experimental doses and the brief exposure to a laboratory discriminating dose is not known. However, the lack of strong selection might be explained if the treatment had been sufficient to kill any heterozygotes as well as susceptible homozygotes. Mortality would be independent of the resistance genotype, and so survival would depend upon escaping a fatal dose of insecticide in the bin.

In this model of a fabric treatment with insecticide, a beetle is either in a refuge or in transit to another. The untreated grain itself may be considered as a very large refuge. Only while it is in transit may a beetle encounter a fatal dose of insecticide. Infestation of the grain will depend on the effectiveness of the fabric treatment as a *cordon sanitaire*, killing beetles moving from their original refuge into the grain. Beetles can disperse freely in the grain bins, so we would expect the efficacy of the control strategy to be related to the fraction of the surface that has been treated. This expectation is borne out by the size of refuge populations, and to a lesser extent by the grain populations.

Refuges were able to sustain beetles even in the most extensively treated bins. They acted as foci of infestation, enabling a strain containing a high proportion of susceptible individuals to colonise a central bulk of grain. If refuges can act as foci of reinfestation in grain stores then the frequency of resistance alleles in refuge populations will be crucial to the rate at which resistance evolves and control failure occurs when beetles migrate into a new grain bulk. This frequency will depend on the relative fitness of each genotype over the range of insecticide doses encountered, and the absolute rate of migration between refuges and grain.

The application of a dose sufficient to kill heterozygotes, while allowing the maximum acceptable number of individuals (which will be mainly homozygous susceptibles, initially) to escape exposure has been suggested as a strategy for delaying the spread of resistance, while maintaining control of pest numbers. In this study, the frequency of resistant beetles in the refuges appeared to increase as the treatment became more extensive, as would

be expected under such a strategy. However, this strategy does not account for the apparent increase in the proportion of susceptible beetles in refuge populations compared with those of the original cultures and of the beetles from the grain in the control bin.

The grain bins function well as a model system for studying the response of *O. surinamensis* to insecticide use strategies under controlled, yet realistic, conditions. Their continued use will provide a much needed bridge between our understanding of this beetle as a laboratory organism and as a pest in the grain store by the study of dispersal behaviour, productivity and mortality in the refuges and grain.

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REFERENCES

Caprio, M.A.; Tabashnik, B.E. (1992) Gene flow accelerates local adaptation among finite populations: simulating the evolution of insecticide resistance. *Journal of Economic Entomology*, **85**, 611-620.

Chamberlain, S.J. (1990) Determination of multi-pesticide residues in cereals, cereal products and animal feed using gel-permeation chromatography. *Analyst*, **115**, 1161-1165.

Cogan, P.M.; Wakefield, M.E.; Pinniger, D.B. (1990) PC, a novel and inexpensive trap for the detection of beetle pests at low densities in bulk grain. *Proceedings of the 5th International Working Conference on Stored-Product Protection*, 1321-1328.

Curtis, C.F.; Rawlings, P. (1980) A preliminary study of dispersal and survival of *Anopheles culicifacies* in relation to the possibility of inhibiting the spread of insecticide resistance. *Ecological Entomology*, **5**, 11-17.

Georghiou, G.P.; Taylor, C.E. (1977) Genetic and biological influences in the evolution of insecticide resistance. *Journal of Economic Entomology*, **70**, 319-323.

Muggleton, J; Pinniger, D.B.; Webb, D.P.; Woodward, M.A. (1986) Treatment of a farm grain store with pirimiphos-methyl and the consequences for resistance in saw-toothed grain beetles (*Oryzaephilus surinamensis*). *Brighton Crop Protection Conference - Pests and Diseases 1986*, **2**, 599-606.

Prickett, A.J; Muggleton, J.; Llewellin, J.A. (1990) Insecticide resistance in populations of *Oryzaephilus surinamensis* and *Cryptolestes ferrugineus* from grain stores in England and Wales. *Brighton Crop Protection Conference - Pests and Diseases 1990*, **3**, 1189-1194.

Raymond, M.; Callaghan, A.; Fort, P.; Pasteur, N. (1991) Worldwide migration of amplified resistance genes in mosquitoes. *Nature*, **350**, 151-153.

Taylor, C.E.; Quaglia, F.; Georghiou G.P. (1983) Evolution of resistance to insecticides: a cage study on the influence of migration and insecticide decay rates. *Journal of Economic Entomology*, **76**, 704-707.

Unruh, T.R. (1990) Genetic structure among 18 west coast pear psylla populations: implications for the evolution of resistance. *American Entomologist*, **36**, 37-43.