SESSION 9C

THREATS TO THE EFFICACY OF AGROCHEMICALS – RESISTANCE AND DEGRADATION

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POSTERS 9C-1 to 9C-19

THE SURVIVAL OF *PHYTOPHTHORA INFESTANS* IN POTATO TUBERS - THE INFLUENCE OF PHENYLAMIDE RESISTANCE

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ABSTRACT

Twenty-four isolates of *Phytophthora infestans* (14 phenylamide-resistant and 10 phenylamide-sensitive) from potato crops grown in Northern Ireland between 1980 and 1987 were used to study overwinter survival of the pathogen in tubers. Resistant isolates grew faster than sensitive ones on tuber slices at 5°C and in some cases at 10°C, but not at 15°C. They also infected more inoculated whole tubers than did the sensitive ones. However, fewer visibly-infected tubers inoculated with resistant than with sensitive isolates survived to produce plants the following season because many were invaded by pectolytic bacteria.

INTRODUCTION

Investigations using isolates of *Phytophthora infestans* collected in Northern Ireland during 1986 showed that three phenylamide-resistant isolates grew faster than four phenylamide-sensitive ones on potato tuber slices at 5° and 10°C, but not at higher temperatures (Walker & Cooke, 1988). When inoculated onto whole tubers in an overwintering experiment, the three resistant isolates infected more tubers than did the sensitive ones, but fewer of these tubers survived and produced plants the following season due to invasion by soft-rotting bacteria. This paper reports the behaviour of a larger number of isolates of *P. infestans* collected over an eight year period in Northern Ireland in terms of their growth rates on tuber tissue and their ability to overwinter in whole tubers.

MATERIALS AND METHODS

Isolates of *P. infestans*

The 24 isolates of *P. infestans* were obtained from potato crops grown in Northern Ireland between 1980 and 1987. All isolates were tested on leaf discs for their sensitivity to phenylamides (Cooke, 1986); the final letter of their code numbers indicates sensitivity (S) or resistance (R). Isolates with the prefix "T" were obtained from tubers, other isolates were from foliage. Zoospores were produced from cultures growing on chick peas (15°C, 21 d) by washing off sporangia, chilling at 5°C (1.5 h) and allowing them to warm to room temperature. Concentration was adjusted to 10^4 zoospores/ml.

Effect of temperature on growth of isolates on tuber slices

Surface-sterilised tubers cv. Kerr's Pink were cut into 5 mm thick slices, each of which was inoculated with a *P. infestans* isolate (one 10 μl drop/slice) and incubated at 5, 10 or 15°C (Walker & Cooke, 1988). Areas of growth were measured between 5 and 42 days later, depending on growth rate.

Overwintering experiment

A crop of potatoes cv. Kerr's Pink was grown to provide tubers for the experiment. The foliage was sprayed at 14-day intervals for blight control, half receiving mancozeb alone (Crop A) and half metalaxyl+mancozeb (Crop B). After harvest, washed, blight-free tubers were inoculated with the 24 isolates (64 tubers/crop/isolate) and placed in simulated seed storage as described previously (Walker & Cooke, 1988). A randomised block design with eight replicates of eight tubers was used. Six months after inoculation, the percentage of tubers with blight and with bacterial soft rot was recorded. Two tubers from each replicate were cut open; where infection was present, isolation of *P. infestans* was attempted and isolates were tested for phenylamide resistance. The remaining tubers were sprouted; tubers which had completely rotted were discarded. Two of the remainder (blighted ones selected preferentially) were planted in pots (125 mm diameter) in a greenhouse (10°C night, 25°C day) with a regular water supply onto capillary matting. The remaining tubers were grown in field plots. Emergence and disease symptoms were recorded.

RESULTS

TABLE 1. The effect of temperature on the growth of *Phytophthora infestans* isolates on tuber slices.

Isolate	Area of growth (cm^2)		
	5°C[a]	10°C[b]	15°C[c]
Experiment 1			
98/87R	2.4	24.1	10.8
T6/81R	2.1	2.5	2.6
T29/81R	1.9	2.9	2.1
2/82R	1.9	2.5	3.7
99/87S	0.8	6.2	3.8
T28/81S	0.8	4.7	3.0
9/83S	0.6	4.9	3.2
T21/81S	0.4	14.1	2.2
S.E. (63 D.F.)	0.19***	1.15***	0.39***
Experiment 2			
46/87R	2.2	11.8	2.2
T28/86R	1.8	12.8	3.7
TC7/87R	1.5	4.6	2.3
T29/86R	1.5	2.6	2.0
88/81R	1.4	3.2	1.4
T8/87S	0.6	1.5	1.7
1/87S	0.5	1.4	1.9
T11/85R	0.4	2.0	0.7
S.E. (63 D.F.)	0.16***	0.53***	0.27***

*** = $P<0.001$
a, b, c measured after 42, 18 and 5 d, respectively

Effect of temperature on growth of isolates on tuber slices

Sixteen of the *P. infestans* isolates were included in two experiments; the behaviour of the remaining eight isolates has already been reported (Walker & Cooke, 1988). In Experiment 1, the four resistant isolates grew faster than the sensitive ones at 5°C, but at 10°C and 15°C growth rates were not consistently related to phenylamide sensitivity (Table 1). In Experiment 2, all but one resistant isolate grew faster than the sensitive ones at 5°C; this trend was also apparent at 10°C, but not at 15°C.

Overwintering experiment

TABLE 2. The survival to planting of potato tubers cv. Kerr's Pink inoculated with isolates of *Phytophthora infestans*.

Isolate	Crop A tubers			Crop B tubers		
	Blighted (%)[a]	Soft-rotted (%)[a]	Blighted & planted (%)[b]	Blighted (%)[a]	Soft-rotted (%)[a]	Blighted & planted (%)[b]
T28/86R	98	97	2	100	100	2
98/87R	98	98	0	98	98	0
46/87R	98	98	8	95	94	4
T29/86R	97	97	0	100	97	8
TC7/87R	97	97	4	92	91	2
T11/85R	92	50	98	33	14	31
2/82R	34	8	35	13	2	15
88/81R	33	28	21	13	13	4
67/86R	16	13	4	8	8	2
T6/81R	13	13	0	6	6	0
64/86R	9	9	0	9	8	2
T29/81R	6	6	0	11	8	6
82/86R	6	6	0	6	6	2
T10/86R	3	3	0	16	13	4
96/86S	83	66	35	5	5	0
1/87S	44	34	13	11	11	2
9/83S	31	9	33	11	11	0
49/86S	13	11	0	11	11	0
99/87S	13	11	6	3	2	2
T8/87S	11	8	8	9	6	6
51/86S	9	8	2	13	13	0
27/86S	6	6	2	20	19	6
T28/81S	6	6	0	13	13	0
T21/81S	5	3	2	14	14	2

S.E. (322 D.F.)	Blight	Soft-rot	Planted
Crop	1.3**	0.9**	0.8***
Isolate	2.8***	2.9***	2.3***
Crop x isolate	4.1***	4.2***	3.3***

** = $P<0.01$ *** = $P<0.001$ a based on 8 tubers/plot

b after discarding totally rotted tubers; based on 6 tubers/plot (2 tubers/plot removed for isolation)

TABLE 3. Emergence of tubers cv. Kerr's Pink inoculated with isolates of *Phytophthora infestans* and planted under glass.

Isolate	Crop A		Crop B	
	Total tubers planted emergence(%)[a]	Blighted tubers planted emergence(%)[b]	Total tubers tubers emergence(%)[a]	Blighted tubers planted emergence(%)[a]
T28/86R	55(0.3)	0(0.1)	0(0.1)	0(0.1)
98/87R	3(0.1)	-(0.0)	96(0.1)	-(0.0)
46/87R	0(0.5)	0(0.4)	40(0.6)	50(0.3)
T29/86R	94(0.3)	-(0.0)	14(0.5)	14(0.5)
TC7/87R	48(0.4)	0(0.3)	48(0.8)	0(0.1)
T11/85R	0	0(2.0)	38	25(1.4)
2/82R	50	31(1.6)	75	50(0.8)
88/81R	56	25(1.1)	88	0(0.3)
67/86R	88	50(0.3)	94	100(0.1)
T6/81R	94	-(0.0)	94	-(0.0)
64/86R	100	-(0.0)	94	100(0.1)
T29/81R	100	-(0.0)	75	33(0.4)
82/86R	100	-(0.0)	88	0(0.1)
T10/86R	94	-(0.0)	94	94(0.1)
96/86S	19	13(1.8)	88	-(0.0)
1/87S	72(1.8)	60(0.8)	94	0(0.1)
9/83S	75	63(1.6)	94	-(0.0)
49/86S	100	-(0.0)	94	-(0.0)
99/87S	100	100(0.4)	88	100(0.1)
T8/87S	100	100(0.5)	100	100(0.4)
51/86S	100	100(0.1)	100	-(0.0)
27/86S	100	100(0.1)	81	33(0.4)
T28/81S	100	-(0.0)	100	-(0.0)
T21/81S	88	0(0.1)	88	100(0.1)

S.E. (322 D.F.)	Emergence (total)	Emergence (blighted)
Crop	3.0 ns	
Isolate	5.7 ***	not analysed
Crop x isolate	8.4 ***	due to missing values

ns = $P>0.05$ *** = $P<0.001$

a based on 2 tubers planted per plot; if fewer survived the mean number per plot is given in parentheses

b the mean number of blighted tubers planted per plot is given in parentheses

All isolates infected some tubers in both crops and soft rot developed in some of the tubers infected by each isolate (Table 2). There was a significant correlation (r = 0.98) between the percentage of blighted tubers and the percentage with soft rot. The five isolates which infected the most tubers were all phenylamide-resistant and these tubers also had the most soft rot. Isolation of *P. infestans* from cut tubers after six months storage proved difficult due to bacterial contamination and was successful in only three cases; the phenylamide sensitivity of these was the same as that of the original inoculum. With every isolate, some tubers from each

crop survived for planting, but with nine isolates in Crop A and seven in Crop B, only tubers without blight symptoms survived. Overall, of tubers inoculated with resistant isolates, 12.4% of those from Crop A and 6.0% of those from Crop B that were visibly infected, survived to be planted. Of tubers inoculated with sensitive isolates, the corresponding figures were 10.2% and 1.9% from Crop A and Crop B, respectively. However, after planting in the greenhouse (Table 3), the percentage of blighted tubers which produced plants was less where they had been inoculated with resistant isolates (16 and 32% emergence from blighted tubers for Crops A and B, respectively) rather than with sensitive isolates (52 and 67% emergence for Crops A and B, respectively). In the field, the pattern of emergence was similar; the data are not included here, but are given by Walker (1990). No foliage infection developed on plants in the greenhouse and no symptoms were seen in the field until after infection was present in adjacent trials.

DISCUSSION

On tuber slices incubated at 5°C, nine out of ten of the phenylamide-resistant isolates tested grew faster than the six sensitive ones. Similarly, in work previously reported (Walker & Cooke, 1988), at low temperature, three out of four resistant isolates from 1986 grew faster than four sensitive ones. In the present study there was less evidence of differences at 10°C than in the earlier work.

In the overwintering trial, it was expected that the sensitive isolates would infect more tubers from Crop A than from Crop B, since Crop B had received foliar sprays containing metalaxyl, which reduces tuber infection by phenylamide-sensitive *P. infestans* (Bruin *et al.*, 1982). However, whilst this was true overall (Crops A and B were infected 22 and 11%, respectively), there was much variation between isolates with some apparently unaffected by the crop treatment. With the resistant isolates there was less difference in tuber infection between the two crops (50 and 43% for A and B respectively).

Overall, resistant isolates infected more tubers than did sensitive isolates and more visibly-infected tubers inoculated with resistant isolates survived to be planted (reflecting both the initial infection rate and the fact that all except totally rotted tubers were planted). However, the proportion of blighted tubers which produced plants was greater where they were infected with sensitive rather than resistant isolates. Thus, as in the earlier trial, a greater potential inoculum source was provided by the sensitive isolates. It was unfortunately impossible to relate this to primary lesions on foliage, since none developed, despite the use of greenhouse conditions claimed to result in formation of lesions on foliage from infected tubers (Keay, 1953).

The greater loss of tubers infected with resistant isolates may be related to their faster growth through tuber tissue at low temperature. This produced more extensive colonisation of tuber tissue by *P. infestans*, which in turn predisposed it to greater development of secondary bacterial soft rot. The surprisingly high survival rate of tubers inoculated with isolate T11/85R may be due to its slower growth rate in or on tuber tissue, as seen in the tuber slice experiment. However, the interaction of *P. infestans* with soft-rotting bacteria such as *Erwinia carotovora* ssp. *atroseptica* (Eca) may be influenced not only by the extent of colonisation of tuber tissue by *P. infestans*, but also by the particular *P. infestans*

isolate involved. Thus studies with excised tuber discs suggest that isolates differ in their inherent abilities to enhance tissue maceration by Eca (Walker, 1990).

Variability between isolates was very evident in this work and this emphasises not only the importance of using as many isolates as possible in studies of survival and fitness, but also the potential for variation in the *P. infestans* population. The tendency of strains of *P. infestans* to grow faster at low temperatures and to fail to survive the winter in infected tubers appears related to phenylamide resistance, but genetic studies would be necessary to ascertain if the two characters are actually linked. Whilst this poorer survival overwinter may help to reduce the proportion of resistant *P. infestans* available to initiate epidemics the following season, it is insufficient to over-ride a strong selection pressure in favour of resistance during the growing season, such as was experienced in Northern Ireland between 1985 and 1988 (Cooke, 1990). Moreover, the possibility cannot be discounted that the ability to develop faster at lower temperatures may be positively beneficial for the spread of foliage infection at the start of the season when night temperatures are limiting.

ACKNOWLEDGEMENTS

The Ministry of Agriculture, Fisheries and Food is thanked for providing a research studentship for A.S.L. Walker.

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THE DEVELOPMENT OF A THREE-WAY FUNGICIDE MIXTURE BASED ON SYNERGISTIC INTERACTION, FOR EFFECTIVE CONTROL OF PHYTOPHTHORA INFESTANS

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ABSTRACT

Over the past seven years Sandoz have studied the concept of synergy as an anti-resistance strategy for the control of potato blight. A synergistic combination based on the phenylamide oxadixyl mixed with cymoxanil and mancozeb has been evaluated in a series of trials in the U.K. and France. This combination has been found to provide good control of potato blight while the crop is actively growing even in the presence of phenylamide-resistant strains and can be recommended for application at 14-day intervals. The benefits of such a combination containing cymoxanil for extending the duration of disease control and to reduce the selection of phenylamide-resistant strains of potato blight are discussed.

INTRODUCTION

The discovery of the phenylamide fungicides which show systemic activity against the Peronosporales, which include the potato blight pathogen Phytophthora infestans, represented a major advance in the control of potato blight. The two-way combination of a contact fungicide such as a dithiocarbamate with a systemic phenylamide fungicide, introduced ten years ago, added significantly to the armoury of fungicides available. For the first time protection of foliage produced between spray applications was possible.

In many cases, throughout Europe and elsewhere, where phenylamides have been used intensively to control diseases, resistant strains of pathogens have now emerged (Davidse, 1982). Anti-resistance measures have been introduced in an attempt to maintain effective control; these include a restriction on the number of applications and a cut off date beyond which such products are not recommended for use.

Over the past seven years Sandoz research workers have studied an alternative anti-resistance strategy for potato blight based on the concept of synergy (Gisi et al., 1985). Synergism is defined as simultaneous actions in which the response of an organism to a mixture is greater than the sum of the individual components. The phenomenon of synergy is well known with herbicides and insecticides but the interaction between components in a fungicidal combination had not been widely studied.

The synergism between the phenylamide oxadixyl and cymoxanil and mancozeb has been demonstrated by research workers studying mycelial growth in the laboratory and on the development of disease in the greenhouse and in the field (Samoucha & Cohen, 1988). Experiments have shown that a ratio of 1 part oxadixyl: 7 parts mancozeb: 0.4 parts cymoxanil optimised synergy and

gave the best levels of control of both phenylamide sensitive and resistant strains. The synergy factor has been shown to be higher for resistant strains than sensitive strains (Grabski & Gisi, 1987).

Research workers in Israel have demonstrated that fungicidal mixtures in general provide more effective control of both phenylamide sensitive and resistant strains of P. infestans and that a mixture of oxadixyl and mancozeb plus cymoxanil was highly effective and synergistic in controlling phenylamide resistant isolates of the late blight fungus. This three-way combination was also found to give a longer duration of control against resistant strains than a two-way combination (Samoucha et al., 1988).

Field experiments have now been carried out in the U.K. and France to evaluate the three-way combination based on oxadixyl, cymoxanil and mancozeb, registered in the U.K. as 'Trustan' and 'Ripost'.

METHODS AND MATERIALS

Four trials were carried out in 1988, two in the U.K. and two in France.

U.K.

Both sites were located in Suffolk. The trials were of a randomised block design with four replicates. Each plot was 3.00 m x 8.00 m comprising 4 rows.

The first trial on cv. Maris Piper was a conventional trial dependent on natural infection.

The second trial in cv. King Edward was inoculated with both phenylamide sensitive and resistant strains to give a combined overall percentage of 40% of phenylamide resistant blight. The inoculum was applied to guard rows planted between treatments and blocks 14 days after the first spray application and again 7 days later.

The treatments applied at both sites were:-

mancozeb at 1360 g AI/ha at 10 and 14 day intervals

metalaxyl + mancozeb at 150 + 1350 g AI/ha at 14 day intervals

oxadixyl + cymoxanil + mancozeb at 200 + 80 + 1400 g AI/ha at 14 day intervals.

Detailed assessments of foliar blight were made every 7 days using the ADAS key 2.1.1.

France

At two sites cv. Bintje was artificially inoculated. The trials were of a randomised block design with four replicates. Each plot was 4 rows x 10.00 m. The inoculum in each case was applied to a guard row between each treatment. Both trials were inoculated 2-4 days after the first application of fungicide. One site was inoculated with 100% phenylamide-resistant strains and the other with 10% resistant and 90% sensitive strains.

The treatments applied at both sites were:

mancozeb at 1600 g AI/ha at 7 day intervals

oxadixyl at 200 g AI/ha at 14 day intervals

oxadixyl + mancozeb at 200 + 1400 g AI/ha at 14 day intervals

oxadixyl + cymoxanil + mancozeb at 200 + 80 + 1400 g AI/ha at 14 day intervals.

Detailed assessments of foliar blight were made every 7 days using the Method No. 6 of the Commission des Essais Biologiques. (C.E.B.).

All trials were treated with a small plot knapsack sprayer using a spray volume equivalent to 400 litres per hectare.

RESULTS

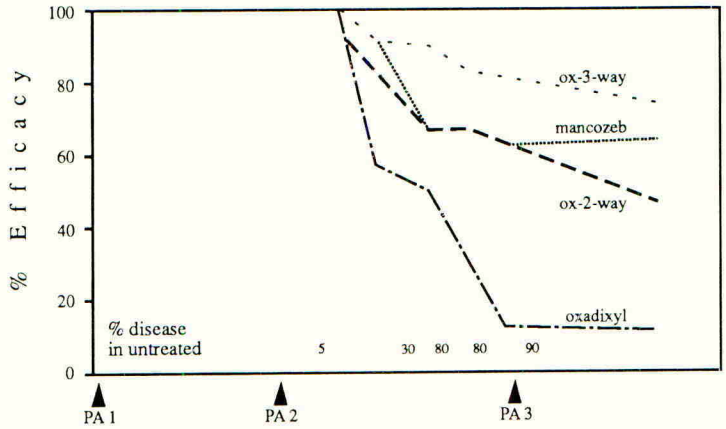
TABLE 1. Control of Phytophthora infestans on potatoes cv. Maris Piper

Fungicide treatment	Rate (g AI/ha)	Spray Interval (days)	% Blight Infection 1/8	8/8	16/8	22/8	30/8	5/9
Mancozeb	1360	10	0.1	0.2	0.6	0.1	6.3	12.5
Metalaxyl + mancozeb	150 + 1350	10	0.1	0.1	1.1	1.1	10.0	10.0
Mancozeb	1360	14	2.0	1.0	1.7	5.4	15.0	18.8
Metalaxyl + mancozeb	150 + 1350	14	1.3	0.8	2.2	7.2	15.0	22.5
Oxadixyl + cymoxanil + mancozeb	200 + 80 + 1400	14	0.5	0.3	1.5	1.0	11.3	12.5
Untreated	-	-	8.1	16.3	42.5	86.2	95.0	98.7

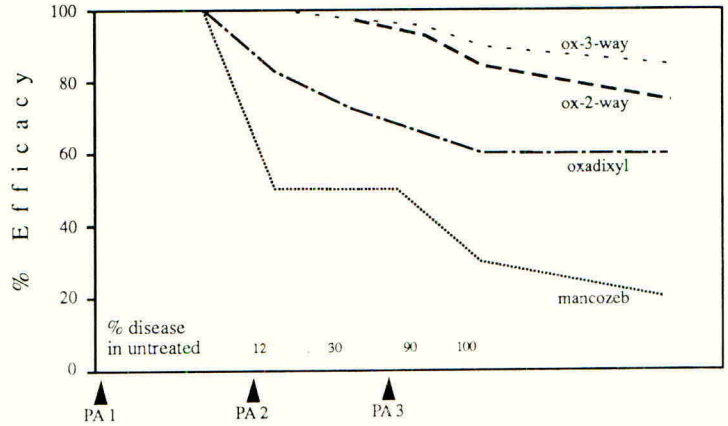
TABLE 2. Control of Phytophthora infestans on potatoes cv. King Edward

Fungicide treatment	Rate (g AI/ha)	Spray Interval (days)	% Blight Infection 22/7	27/7	5/8	12/8	19/8	26/8
Mancozeb	1360	10	0	0.7	9.3	20.3	32.5	45.0
Metalaxyl + mancozeb	150 + 1350	10	0	0.1	3.0	11.0	15.3	20.0
Mancozeb	1360	14	0.1	1.2	11.5	23.0	58.8	73.8
Metalaxyl + mancozeb	150 + 1350	14	0	0.4	6.0	23.0	40.0	49.3
Oxadixyl + cymoxanil + mancozeb	200 + 80 + 1400	14	0	0.1	4.5	13.0	18.0	26.3
Untreated	-	-	8.3	53.8	91.8	99.5	100	100

FIGURE 1. Efficacy of phenylamide mixtures against P. infestans in France. a) 100% resistant strains



b) 10% resistant and 90% sensitive strains



Phenylamide (PA) application every 14 days;Mancozeb application every 7 days.

The two trials in the U.K. (Tables 1 & 2) demonstrated that the three-way combination of oxadixyl with cymoxanil and mancozeb performed better than the two-way combination of phenylamide plus mancozeb, both treatments applied at 14 day intervals. The three-way mixture provided good blight control throughout the season whereas the control with the two-way mixture declined much faster.

The three-way mixture outperformed mancozeb when applied at 14 day intervals and was as effective as mancozeb applied at 10 days at the 'naturally infected' site (Table 1) and was superior at the 'inoculated' site (Table 2). Metalaxyl + mancozeb applied at 14 days was not as effective as mancozeb applied at 10 days; this combination gave similar or slightly better control than mancozeb at 14 days.

In the two trials conducted in France the three-way combination applied at 14 day spray intervals performed better than the two-way

oxadixyl + mancozeb mixture applied at the same spray interval and was superior to mancozeb applied at 7 day intervals.

In the trial inoculated with 100% phenylamide-resistant strains the plots treated with oxadixyl alone were infected to a similar extent to the untreated plots after three applications (Figure 1). The difference in effectiveness between the three-way and two-way combinations occurred after the second application and increased dramatically after the third.

In the second trial, conducted with 10% phenylamide - resistant strains, the level of control achieved by the oxadixyl alone was greater than in the previous trial although effectiveness declined after two applications. In this trial the difference in efficacy between the three and two-way combinations occurred after the third application.

DISCUSSION

Experiments carried out in France and the U.K. confirmed the results found elsewhere. The efficacy of the three-way phenylamide mixture against P. infestans on potatoes was always better than two-way mixtures applied at the same spray intervals. The differences between three-way and two-way mixtures were small at the beginning of the growing season and increased with the number of applications. The superiority of three-way mixtures over two-way mixtures was greater in situations with highly resistant blight populations.

The three-way combination also gave a longer duration of control even where resistant strains were present than cymoxanil + mancozeb under greenhouse and field conditions (Samoucha & Gisi, 1987; Samoucha & Cohen, 1988).

Although cymoxanil is described as a local systemic fungicide, experiments have demonstrated that a foliar application of cymoxanil penetrates into stems and leaves and is translocated acropetally and translaminarly to untreated plant parts. Basipetal translocation from upper to lower leaves as well as lateral movement has also been observed, although to a lesser extent. Cymoxanil is, however, broken down in plant tissues and loses activity under field conditions within 6 days of application (Douchet et al., 1977). Nevertheless, when it is combined with oxadixyl and mancozeb the duration of disease control can be prolonged substantially even in situations where phenylamide resistant sub-populations are present (Samoucha & Gisi, 1987).

The three-way combination has a lower selection pressure for selecting out phenylamide-resistant blight compared to the two-way phenylamide mixture. Increasing the number of spray applications does not build up selection pressure in the same way as the two-way mixture (Cohen and Samoucha, 1989).

Very little is currently known about the mechanism of synergism of fungicides but several hypotheses have been proposed (Gisi, 1989):-

i) increased uptake and binding could lead to higher concentrations in the target cell.

ii) decreased biodegradation in fungal and plant cells may lead to longer duration of activity.

iii) different sites of action of fungicides both in the fungal cell and in the life cycle of the fungus may lead to longer duration of activity. For example, in the three-way mixture oxadixyl provides systemic activity and acts on RNA polymerase preventing formation of haustoria, mancozeb acts at several sites related to cellular respiration during spore germination, while cymoxanil acts on cell permeability at the mycelium stage.

CONCLUSION

The combination of cymoxanil with oxadixyl and mancozeb provides a synergistic mixture. Used in a protectant programme the combination continues to provide rainfastness and protection of new growth even in the presence of phenylamide-resistant strains.

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PHENYLAMIDE USE ON SCOTTISH SEED POTATO CROPS AND RESISTANCE IN *PHYTOPHTHORA INFESTANS* ISOLATED FROM PROGENY TUBERS

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ABSTRACT

Phenylamide resistance in *Phytophthora infestans* isolated from tubers of classified seed potato stocks during the 1988/1989 storage season was widespread in the main seed growing areas of Scotland and at a relatively high incidence (57% of crops tested). For crops in which resistance was detected, a high percentage of tubers yielded resistant isolates. Resistant *P. infestans* was detected in March and April and there was little evidence that its incidence declined towards the time of planting. Resistant *P. infestans* was more readily detected in crops which had been treated with a phenylamide+dithiocarbamate product applied at an interval beyond the recommended interval for the dithiocarbamate than where these products were applied at intervals appropriate for the dithiocarbamate. A survey of blight fungicide programmes in Scotland in 1988 identified that approximately 85% of crops were not adequately protected during the growing season. The principle times at which programmes were inadequate were the switch from a phenylamide product to a protectant and at the time of desiccation.

INTRODUCTION

In 1981, the year after phenylamide fungicides were made commercially available in the U.K., resistance in *Phytophthora infestans* to them was first detected on the mainland U.K. in south-west Scotland (Holmes & Channon, 1984). Resistance was found in 44% of crops in south-west Scotland in that year. The incidence of resistance in crops in England and Wales and also Northern Ireland has increased sharply over the years 1987-1989 (Cock & Cooke, personal communications). Phenylamide resistance had not been detected in the main area of Scottish seed potato production, the north and east of Scotland, up to and including the 1987 growing season. The main purpose of this survey was to determine the incidence of phenylamide-resistant *P. infestans* in the seed producing areas. Blight was isolated from seed tubers since tubers are the main source of inoculum for blight outbreaks in subsequent crops, whether they are planted seed, in potato dumps or volunteers, and blighted tubers were readily available in 1988.

METHODS AND MATERIALS

Seed tuber blight survey

All Scottish Seed Potato Development Council (SSPDC)-registered growers were requested during the 1988/89 storage season to check their seed

stocks for tuber blight, to send a sample of 25 blighted tubers from one stock for testing and to complete a questionnaire covering crop details, in particular, the blight fungicide spray programme. Thirty-five samples were received, three had no blighted tubers, P. infestans was isolated and tested for phenylamide resistance from 28 and blight could not be isolated from four samples.

On receipt, blighted but otherwise scund tubers were washed individually under running tap water, air-dried and then surface sterilised by dipping in industrial methylated spirit. Two pieces of blighted tissue were removed from each tuber and aseptically transferred onto rye B agar (Caten & Jinks, 1968) amended with rifamycin, pimaricin and benomyl (R I Price, ADAS, Trawsgoed, personal communication). The isolates of P. infestans were then bulked on detached leaves of cultivar Maris Piper to provide sufficient inoculum for resistance testing. Isolates were tested for resistance to 100 ppm metalaxyl using the floating potato leaf disc method (Carter et al.,1982). For most crops all of the isolated blight was bulked to produce one sample for testing, but for eight crops, which initial tests demonstrated to have resistant blight, separate tests were conducted on the isolates from individual tubers to establish the distribution of phenylamide-resistance within stocks.

RESULTS

The incidence of phenylamide-resistant P. infestans

Crops from nine of the 11 counties from which samples were received contained resistant P. infestans (Table 1). Phenylamide-resistant P. infestans was detected in 16 (57%) of the 28 crops tested.

TABLE 1. Resistance to phenylamides in P. infestans from tubers of classified seed potato crops produced in different Scottish counties in 1988

County	Number of crops tested	Number of crops in which resistant P. infestans detected
Angus	5	3
Perth	5	4
Aberdeen	6	2
Kincardine	2	1
Fife	2	1
Banff	1	0
Moray	2	0
Dumfries	1	1
Roxburgh	2	2
East Lothian	1	1
Stirling	1	1
Total	28	16

For the eight crops which initial tests showed to contain resistant blight an average of 71% of tubers were infected with resistant P. infestans (Table 2). Resistant blight was readily detected in the latter half of the storage season (Table 3) and there was no evidence that resistant strains were scarce in March and April (Tables 2 and 3).

TABLE 2. The incidence of phenylamide-resistant P. infestans in individual tubers from eight Scottish crops

Crop number	Month P. infestans isolated	Number of tubers tested	Percentage of tubers with resistant P. infestans
12	January	7	100
23	February	8	75
27	February	9	100
28	February	4	75
31	February	3	67
32	March	12	75
33	March	10	40
34	March	6	33

TABLE 3. The incidence of phenylamide-resistant P. infestans in seed tubers in relation to storage period

Month of isolation of P. infestans	Number of crops yielding phenylamide-resistant P. infestans (number of crops tested)
January	5(7)
February	8(17)
March	3(3)
April	0(1)

Resistance was more prevalent where phenylamide+dithiocarbamate mixtures had been applied at intervals greater than those recommended for the dithiocarbamate alone compared with when they were either not applied or were sprayed at the appropriate intervals for the dithiocarbamate (Table 4). However, these data should be regarded as preliminary since only a relatively small percentage of crops in the survey were not treated with such mixtures at extended intervals.

Adequacy of blight fungicide programmes

For 33 crops, full details of the blight fungicide programmes used, giving dates of application and products, were provided. Details of the blight high risk periods for individual crops were not available therefore the suitability of spray intervals was judged against the maximum

recommended spray intervals for the products used and there is therefore a bias in favour of the programmes. Furthermore, three of the fungicides used, i.e. metalaxyl + mancozeb, oxadixyl + mancozeb and benalaxyl + mancozeb, had recommendations for 21 day intervals at the start of the season prior to high risk conditions occurring. Such intervals are now generally considered to be too long but they were not counted as extended in this survey.

TABLE 4. The incidence of phenylamide-resistant *P. infestans* in tubers in relation to the blight fungicide programme applied to the parent crop

	Number of crops from which *P. infestans* isolated was: resistant	sensitive
Phenylamide+dithiocarbamate product used and appropriate interval between dithiocarbamate sprays exceeded	12	4
Phenylamide+dithiocarbamate product used but dithiocarbamate interval not exceeded	2	2
Phenylamide product not used	1	2

The interval between two applications of fungicide was extended for 25 out of the 33 crops (76%). For 52% of the 25 the extension occurred between the last phenylamide application and the switch to a protectant towards the end of the season. For 32% the extension was between earlier fungicide sprays, and for 16% of crops there were extensions for both. Twenty crops had one extended interval, five had two and one had three. The majority of crops (19) in which there were extensions to spray intervals had extensions of five days or less but 10 and four crops had extensions of between six and 10 and between 11 and 15 days respectively.

In 14 out of 33 crops (42%) the interval between application of the last fungicide spray and of the desiccant was too long. As before, the majority of crops had extensions of between one and five days but for four crops the extensions were between six and 12 days.

Sulphuric acid was used to desiccate 18 crops but only eight of these were treated within 14 days of the last fungicide application. Diquat was used to desiccate 14 crops, eight of which were desiccated within 14 days of the last fungicide application. However, because of the slower haulm destruction with diquat compared with sulphuric acid, eight crops out of 14 were not adequately protected from blight during desiccation.

DISCUSSION

Phenylamide-resistant *P. infestans* was shown by this survey to be widespread in the main seed producing areas of Scotland. Gilmour (personal

communication) found that 85% of foliar blight samples collected from the east and north of Scotland in 1988 contained resistant P. infestans. There is legitimate concern over the contribution of phenylamides to blight control because high incidences of phenylamide resistance have been detected previously in other parts of the UK by ADAS and DANI. However, it must be taken into account that the resistance test used currently can only indicate whether phenylamide-resistant blight is present or not in a sample, it cannot determine whether all or a very low percentage of the sporangia in a sample are resistant. The contribution of phenylamide+dithiocarbamate products to control will clearly depend on the proportion of phenylamide-sensitive blight present prior to fungicide application to the crop and therefore the contribution cannot be predicted until this is known.

It is known at present that resistance to phenylamides is widespread in the UK and the results of this survey suggest that the incidence is likely to be high at the start of the growing season. Since it is impossible at present to predict the proportion of resistance in any area then if phenylamide+dithiocarbamate products are used to control blight they should be applied at the intervals recommended for the dithiocarbamate component when used on its own. There should be no increased risk of loss of control compared with the use of dithiocarbamate alone when phenylamide mixtures are applied at these intervals, even where a significant proportion of resistance occurs. Where the incidence of resistance is low there is likely to be a benefit from the use of products which contain a phenylamide. The major disadvantage of the continued use of phenylamide+dithiocarbamate mixtures is that selection pressure for resistant forms continues and the contribution of phenylamides to blight control may diminish further. Preliminary evidence from this survey suggests that phenylamide resistance is more likely to occur in crops where phenylamide+dithiocarbamate mixtures have been applied at intervals beyond those recommended for the dithiocarbamate.

The extension of spray intervals, irrespective of the type of fungicide used, is a more likely cause of loss of control than the use of phenylamide two-way mixtures. Eighty-five percent of the crops surveyed were not adequately protected against blight either between fungicide sprays, between the application of the last fungicide spray and haulm desiccation or during the desiccation period. The inadequate protection can be partly explained by weather conditions unsuitable for spraying at critical times in 1988 - July of 1988 was particularly wet in many of the areas covered by this survey - but not all the extended intervals can be explained by this. Experience in blight fungicide trials over many years at the Scottish Agricultural College (Auchincruive) has shown that the recommended intervals for blight fungicides have to be strictly adhered to. The protection of crops against blight during the period after diquat application also requires improvement.

ACKNOWLEDGEMENTS

We thank Dr J Gilmour, Crop Protection Department, Scottish Agricultural College (Edinburgh) for co-ordinating requests for tuber samples and questionnaires; Mrs L Raynor and Miss S Mohammed, Plant Sciences Department, Scottish Agricultural College (Auchincruive) for technical assistance, the SSPDC for financial support and the growers for samples.

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DECREASED SENSITIVITY TO FENARIMOL IN ITALIAN POPULATIONS OF UNCINULA NECATRIX

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(NO WRITTEN SUBMISSION)

DMI RESISTANCE AND STEROL 14α DEMETHYLATION IN *RHYNCHOSPORIUM SECALIS*

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ABSTRACT

Cross-sensitivity patterns to three DMI fungicides were established using eight *Rhynchosporium secalis* strains differing 200-300 fold in their sensitivity to triadimenol. In most strains cross-resistance occurred between triadimenol, propiconazole and prochloraz, but the resistance levels for propiconazole, and particularly prochloraz, were always lower. All three fungicides inhibited sterol 14α demethylase but higher inhibiting doses were required by resistant strains compared with sensitive strains. Triadimenol sensitivity did not correlate with any differences in metabolism or uptake of this fungicide. It is suggested that resistance to triadimenol and other DMI fungicides arises through alteration of the target sterol 14α demethylase, but that propiconazole and prochloraz retain sufficient activity to remain effective. Consequently, both prochloraz and propiconazole should continue to give good disease control in practice, providing additional resistance mechanisms are not selected.

INTRODUCTION

Fungicides that inhibit the lanosterol 14α demethylase (14DM) step in sterol biosynthesis (DMIs), have now been used for more than 10 years to control barley leaf blotch, caused by *Rhynchosporium secalis*. During this period we have monitored the sensitivity of *R. secalis* to these fungicides, and have isolated field strains differing by at least 100-fold in their sensitivity to triadimenol, and somewhat less to propiconazole (Girling *et al.*, 1988). Cross sensitivity patterns for DMIs can vary, and the level of resistance to one may differ considerably from that of another, even within the triazole group of DMIs (Kendall, 1986). Consequently, where resistance develops to one DMI, there may still be scope to use others in strategies aimed at combating further development of resistance. An understanding of the biochemical mechanisms involved would greatly assist this approach.

More efficient efflux of DMI fungicides can account for resistance in laboratory mutants of some fungi (De Waard & van Nistelrooy, 1988), but other mechanisms may operate (Hollomon *et al.*, 1990). Detoxification, failure to activate, deposition in lipid droplets, and reduced fungicide binding to the target 14DM, may contribute. Perhaps the best understood mechanism of resistance relates to how accumulation of otherwise toxic sterols is prevented. Triazole resistant *Saccharomyces cerevisiae* mutants invariably have a genetic block in the Δ^{5-6} desaturase (Taylor *et al.*, 1983; Watson *et al.*, 1989). This prevents accumulation of the toxic 14 methyl

ergosta $\Delta^{8,24(28)}$ dien-3β 6α diol which occurs in treated sensitive strains, and the 14 methyl fecosterol that accumulates instead, at least fulfils the bulk function of sterols (Parks *et al.*, 1986).

In *R. secalis*, DMI resistance was not associated with alterations in sterol content of untreated mycelium, and toxic diols were not detected in a treated wild-type strain, although some effects on sterol biosynthesis after the demethylation steps were observed (Girling, 1988). In this paper we extend our studies on the mechanism of resistance to DMI fungicides in *R. secalis* to include the imidazole, prochloraz, as well as other triazole fungicides.

METHODS

R. secalis strains

Eight strains (Table 1) originating as single spore isolates from field samples collected during a UK survey of fungicide sensitivity, were maintained on Czapek-Dox agar with the addition of 0.5% (w/v) mycological peptone. Longer-term storage was as spore suspensions in 1% (w/v) sterilised skim milk solution on silica gel.

TABLE 1. Sensitivity of eight *Rhynchosporium secalis* strains to DMI fungicides in liquid culture

Strain	Year of isolation	Sensitivity (ED50 μg/ml) Triadimenol	Propiconazole	Prochloraz
588.03	1989	0.016 a*	0.004 a	0.0007 a
553.07	1989	0.031 a	0.039 ab	0.0006 a
554.24	1989	0.040 a	0.030 a	0.006 b
588.11	1989	0.088 ab	0.006 a	0.0001 c
ACP	1986	0.317 b	0.011 a	0.005 b
554.26	1989	2.50 c	0.21 bc	0.026 d
588.05	1989	10.60 c	0.61 c	0.037 d
243.02	1986	19.09 c	0.11 bc	0.006 b

* Values in vertical columns followed by the same letter are not significantly different (P = 0.05).

Incorporation of [U-^{14}C] acetate into sterols and determination of ED50 values

Flasks containing 50 ml liquid glucose-malt-yeast medium with added mycological peptone (MYMPG), were inoculated with cell suspensions of each strain, giving an initial inoculum density of 5 x 10^4 cells/ml. All fungicides were added from stock solutions in methanol to give a five-fold concentration range, estimated to encompass the ED50 and ED95 values expected for each strain. Triadimenol, propiconazole and prochloraz were

added after autoclaving (120°C) when the medium had cooled to 40°C. Duplicate flasks were prepared for each fungicide concentration plus controls, and shaken at 17°C for 10 days. 1μ Curie of [U-^{14}C] sodium acetate was added to each flask and incubation continued for a further 24 hours. Mycelial pellets were collected by filtration, washed and freeze dried; dry weights were used to calculate both ED50 and ED95 values for each strain x fungicide combination.

Sterol extraction and analysis

Total lipids were extracted by grinding freeze-dried mycelia with acid-washed sand in liquid nitrogen, and extracting with 10ml chloroform: methanol (2:1). Radioactivity in this lipid extract was determined by counting 100μl aliquots in a liquid scintillation spectrometer (LKB 1215 Rackbeta 11). The remainder of each extract was evaporated to dryness and taken up in 1.0 ml [ED50] or 0.1 ml [ED95] hexane. Sterols were separated by TLC on zone concentrating silica gel F^{254} (Merck 11798) using benzene: diethyl ether (9:1) as the solvent. Lanosterol, ergosterol, and squalene (25μg) were applied as standards. Spots were visualised under U/V light (254 nM), and the plates radioautographed (Kodak X-Omat-s) for 7 days to visualise any radioactive spots corresponding to dimethyl (lanosterol), monomethyl and desmethyl (ergosterol) sterols. Each spot was scraped from the plate and its radioactivity determined by liquid scintillation counting.

Uptake of [^{14}C] triadimenol

Flasks containing 50ml MYMPG medium were inoculated with *R. secalis* cell suspensions to give a final concentration of 2 x 10^5 cells/ml, and incubated with constant shaking for 6 days at 17°C. Duplicate flasks including uninoculated controls, were then labelled with 0.2 μCurie of [^{14}C] triadimenol and incubated for a further 48 hours. Mycelium was separated by filtration and washed with distilled water. Filtrates were combined, made up to 100 ml, and aliquots (100 μl) counted to determine radioactivity not taken up by the fungus. After freeze-drying, mycelium was ground with acid-washed sand in liquid nitrogen, extracted with methanol (10 ml), evaporated to dryness and redissolved in 0.1 ml methanol. All of this extract was applied to a TLC plate (Silica gel F^{254} Merck 5715) and separated using diethyl ether:hexane:methanol (72:14:14). A single radioactive spot for each extract was located by radio-autography near the solvent front, and eluted before rechromatographing, together with [^{14}C] triadimenol standard, on Silica gel F^{254} using dichloromethane:isopropanol (90:10) as solvent system. This separated the two triadimenol diastereomers which were eluted separately and radioactivity determined by liquid scintillation counting.

Chemicals

All fungicides were technical grade and kindly supplied by their respective manufacturers. Solvents used were analytical grade and biochemicals were purchased from Sigma, Poole, UK. [U-^{14}C] Sodium acetate (CFA 229 Sp. act. 2.04G B$_q$/m mole) was purchased from Amersham International, and [^{14}C] triadimenol was a gift from Bayer AG.

RESULTS

Cross resistance patterns

Dry weight determinations produced ED50 values slightly higher than those previously reported based on turbidity measurements (Girling *et al.*, 1988). The eight strains selected for study showed a wide range of variation in sensitivity to all three fungicides (Table 1), which reflected the variation we have observed in more extensive monitoring of field populations (Jones, 1990). Some cross-resistance exists between all three fungicides, but the patterns are clearly not consistent across all eight strains (e.g. strains 243.02 and 588.11 differ markedly).

Incorporation of [U-^{14}C] acetate into sterols

All strains incorporated acetate into the overall sterol fraction in the absence of fungicide. Although amounts incorporated over 24 hours varied between 3 and 13 per cent of applied radioactivity depending on the strain, differences were not correlated with DMI sensitivity. Incorporation into lanosterol also varied, but again this was not related to DMI sensitivity (Table 2). At concentrations equivalent to ED50 values, all three fungicides increased incorporation into total sterols. Most of this was reflected by increased incorporation into methyl sterol (lanosterol), confirming that these DMIs did indeed inhibit 14DM in all eight strains of *R. secalis*. However, higher concentrations were needed to achieve these effects in strains with lower sensitivity to DMIs.

TABLE 2. Incorporation of U-^{14}C acetate into lanosterol as a percentage of total incorporation into sterols

	Incorporation into lanosterol (%)*							
Strain	No fungicide	Triadimenol ED_{50}	Triadimenol ED_{95}	Propiconazole ED_{50}	Propiconazole ED_{95}	Prochloraz ED_{50}	Prochloraz ED_{95}
588.03	4.2 ± 1.3[x]	28.9	48.8	NT[+]	NT	4.6	48.9
553.07	8.7 ± 3.1	41.5	49.7	36.7	43.0	23.2	55.6
554.24	13.3 ± 10.0	30.8	58.3	23.5	19.1	16.4	49.8
588.11	5.4 ± 2.8	NT	31.2	NT	NT	NT	43.1
ACP	17.8 ± 2.2	NT	46.7	24.6	49.5	21.2	NT
554.26	6.4 ± 4.4	19.5	27.4	36.9	NT	NT	36
588.05	14.7 ± 3.6	36.8	NT	49.0	NT	28.2	NT
243.02	8.2 ± 3.7	22.8	57.0	14.5	50.4	NT	51.2

* Percentage incorporation into lanosterol was calculated from incorporation into methyl, monomethyl and desmethyl sterols, expressed on the basis of mg dry weight^{-1} of the mycelium used for extraction of sterols.

[+] NT = not tested.

[x] SED (P = 0.05).

Uptake and metabolism of [^{14}C]-triadimenol

Although only three strains were examined, no metabolites of triadimenol were detected during the 48 h incorporation period, and all radioactivity within the mycelium was recovered as triadimenol. Amounts taken up averaged 1% of the radioactivity added to each flask, and were not related to DMI sensitivity. Separation of the triadimenol by TLC suggested that less of the active diastereometer (1*S*2*R*; 1*R*2*S*) was taken up than the inactive one (1*S*2*S*; 1*R*2*R*) but again this was not linked to DMI sensitivity (Table 3).

Table 3. Separation of triadimenol diastereomers recovered from *Rhynchosporium secalis*

	Radioactivity (%)	
Strains	Rf. 0.72 (1*S*2*S*; 1*R*2*R*)	0.69 (1*S*2*R*; 1*R*2*S*)
ACP	26.5	73.3 ± 5.4*
588.05	36.1	63.9 ± 4.8
243.02	28.6	71.4 ± 0.3
Triadimenol standard	17.9	82.1

* SED (P = 0.05).

DISCUSSION

Unlike cereal powdery mildews, in which DMI resistance reflects a shift in an otherwise unimodal, overlapping population (Skylakakis & Hollomon, 1987), *R. secalis* produces two almost distinct groups following selection with DMIs (Jones, 1990). The eight strains examined here were chosen to reflect this bimodal distribution, in order to test the hypothesis that bimodal resistance might relate to a single biochemical mechanism. The two groups differed about 200-300 fold in sensitivity to triadimenol, but somewhat less (20-30 fold) to propiconazole. For prochloraz, which was the most active *in vitro* of these DMIs against *R. secalis*, cross-resistance patterns were less clear.

Differences in uptake and metabolism do not appear to account for resistance to triadimenol, and presumably the other DMIs. Instead, the target enzyme 14DM seems less inhibited in resistant strains than in sensitive ones, and higher doses were simply needed to achieve the same effects as seen with lower doses applied to sensitive strains. There is no suggestion from this work, or our previous studies (Girling *et al.*, 1988) that a defect in the Δ^{5-6} desaturase is involved in resistance. In *R. secalis* propiconazole inhibits Δ^{22} desaturase (Girling *et al.*, 1988) and changes in this enzyme may also contribute to resistance to propiconazole. However, differences in sensitivity to 14DM explain some of the differences in biological activity between triadimenol, propiconazole and prochloraz, including differences in cross-resistance patterns. Whilst 243.02 required

high levels of triadimenol to inhibit 14DM, the same degree of inhibition was achieved with a 1000 fold lower concentration of prochloraz, and this matches the biological performance of these two DMIs against 243.02. Yet with some sensitive strains, similar levels of 14DM inhibition were achieved with almost identical concentrations of all three fungicides. It seems, therefore, that the biochemical basis of triadimenol resistance in *R. secalis* involves some alteration to the target 14DM, and that other DMIs may retain suficient activity against this altered enzyme to provide good disease control. At present, problems of control associated with triadimenol resistance seem widespread in *R. secalis* in the UK, whereas propiconazole and prochloraz resistance have not caused practical problems (Jones, 1990). However, strategies using propiconazole and prochloraz may only remain effective as long as no other resistance mechanism is selected within the *R. secalis* population.

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SENSITIVITY OF RHYNCHOSPORIUM SECALIS TO DMI FUNGICIDES

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ABSTRACT

Sensitivity of Rhynchosporium secalis to DMI fungicides was assessed in vitro for 464 isolates from England and Wales in 1987 and for 151 isolates from south-west England in 1989, and compared with published data for 1981. There was a reduction in sensitivity to triadimenol between 1981 and 1987, and a further reduction by 1989. Sensitivity to propiconazole also declined between 1987 and 1989. In 5 field trials mixtures of DMI fungicides with carbendazim or tridemorph were more effective for control of R. secalis than the DMI fungicide alone.

INTRODUCTION

Leaf blotch, or scald, caused by Rhynchosporium secalis is a common disease of barley throughout the world, particularly in cooler and semi-humid areas (Shipton et al., 1974). In the United Kingdom leaf blotch is common in the wetter western and northern areas, and can cause serious losses on autumn-sown crops and early spring-sown crops. Yield losses of up to 40% have been reported (Jenkins & Jemmett, 1967).

A range of demethylation-inhibiting (DMI) fungicides has activity against R. secalis, including triadimefon, triadimenol, propiconazole, prochloraz, flutriafol, and flusilazole. Many winter barley crops in the United Kingdom have received one or two and in some cases three applications of a DMI fungicide each year since the late 1970's, so there was a distinct possibility that there had been selection for reduced sensitivity to DMI fungicide in R. secalis.

This paper reports the results of surveys in England and Wales in 1987 and 1989 for sensitivity to DMI fungicides in R. secalis, and field trials in 1988 and 1989 on the efficacy of fungicides against R. secalis.

MATERIALS AND METHODS

In vitro studies

Hollomon (1984) reported the development of an in vitro assay for assessing sensitivity of R. secalis to triadimenol, in which the growth of the pathogen was assessed on yeast malt agar (YMA) slants amended with a two-fold dilution series ranging from 3.2 to 0.05 mg/l triadimenol. Triadimenol sensitivity was assessed as the minimum inhibitory concentration (MIC).

In the present study, a modification of this method was used. R. secalis was isolated from washed and surface-sterilised lesions (1-2 minutes in sodium hypochlorite solution producing 3% available chlorine) and plated on potato dextrose agar (PDA) in 9 cm diameter plastic petri dishes. PDA was used for convenience since preliminary studies showed results on PDA and YMA to be comparable. Isolates were maintained in the dark at 18°C and sub-cultured every 14 days. The sensitivity of isolates to fungicides was determined by streaking spores of R. secalis on PDA amended with fungicide at the concentrations listed in Tables 1 and 2. Carbendazim was also tested, at 1.0 mg/l. An unamended control plate was included with each set of tests, and two isolates of known sensitivity were included with all tests to provide a check. The doses of triadimenol were based on the two-fold series of doses used by Hollomon (1984) and the doses of propiconazole in 1987 were those used by Hollomon (pers. comm.). The propiconazole doses were changed to a twofold series in 1988 and 1989 to assess MICs more accurately. The MIC was assessed as the lowest concentration which completely inhibited growth of the fungus into the agar. The single dose of carbendazim was selected because experience with other pathogens indicated that sensitive strains are inhibited by doses considerably below 1.0 mg/l whereas resistant strains show little inhibition by doses of 1.0 mg/l.

A total of 464 isolates of R. secalis were obtained from 95 of the randomly-selected farms included in the 1987 National Survey of Winter Barley Diseases conducted by the Agricultural Development and Advisory Service, and tested for sensitivity. In 1989, 151 isolates from 13 farms in the survey, all in the South-Western Region, were tested. Samples were collected from crops at GS 71-77 (Zadoks et al., 1974).

Field Trials

Three field trials on winter barley were made in 1988, in Somerset and Devon, and two in 1989, in Somerset and Avon. Details of fungicide treatments are given in Table 4. Approximately 30 isolates of R. secalis from each site (isolated prior to spray application) were tested for sensitivity to triadimenol and propiconazole.

RESULTS

Results of the 1987 and 1989 surveys are given in Tables 1 and 2. Results of in vitro tests for sensitivity of the pathogen at the trial sites are in Table 3, and mean results for the 1988 and 1989 field trials are in Table 4.

The 1987 survey (Table 1) showed that the R. secalis population had a bimodal distribution of sensitivity to triadimenol, with 46% of isolates having an MIC of 6.4 mg/l or above. In general, isolates with an MIC of 6.4 or 12.8 mg/l triadimenol had an MIC propiconazole of 0.2 or 1.0 mg/l. All isolates were sensitive to 1.0 mg/l carbendazim. In the 1989 survey (Table 2) 62% of isolates had an MIC triadimenol of 6.4 mg/l or above. 70% of isolates had an MIC propiconazole of 0.2 mg/l or greater, compared with 5% in 1987. All isolates were sensitive to 1.0 mg/l carbendazim.

TABLE 1. 1987 survey of sensitivity to triadimenol and propiconazole of 464 isolates of R. secalis.

MIC triadimenol (mg/l)	% isolates	MIC propiconazole (mg/l)	% isolates
0.8	44	0.008	7
1.6	1	0.04	43
3.2	8	0.2	45
6.4	34	1.0	5
12.8	12		

TABLE 2. 1989 survey of sensitivity to triadimenol and propiconazole of 151 isolates of R. secalis.

MIC triadimenol (mg/l)	% isolates	MIC propiconazole (mg/l)	% isolates
0.08	19	0.05	2
1.6	7	0.1	6
3.2	12	0.2	22
6.4	25	0.4	42
12.8	27	0.8	22
16.6	4	1.6	6
25.6	3		
33.3	3		
51.2	0		

TABLE 3: MIC values for R. secalis isolates from field trial sites.

		Mean MIC values (mg/l) triadimenol	propiconazole
1988	(Site 1	3.7	0.49
	(Site 2	2.7	0.32
	(Site 3	3.7	0.48
1989	(Site 1	1.6	0.15
	(Site 2	1.3	0.09

In the field trials (Table 4), the most effective single sprays for leaf blotch control were flusilazole + carbendazim, propiconazole + carbendazim, propiconazole + tridemorph and prochloraz + carbendazim. Triadimenol, alone or mixed with tridemorph, was less effective than propiconazole or prochloraz. Results at all three sites in 1988 were comparable, as were results at the 1989 sites. Other diseases were of negligible importance. Yield increases in 1988 trials were associated with disease control, but carbendazim gave the smallest increases in yield although it was more effective for disease control than triadimenol or prochloraz.

TABLE 4: Results of 1988 and 1989 field trials.

	Treatment[a]	Rate (kg AI/ha)	1988 (3 trials) Mean % leaf blotch GS75 leaf 1	1988 (3 trials) Mean % leaf blotch GS75 leaf 2	1988 (3 trials) Mean yield (t/ha) (2 trials only)	1989 (2 trials)[b] Mean % leaf blotch GS 59-75 leaf 2	1989 (2 trials)[b] Mean % leaf blotch GS 59-75 leaf 3
1.	Untreated	-	30.1	40.4	5.27	8.2	16.2
2.	Carbendazim	250	11.2	20.8	5.62	1.9	5.4
3.	Triadimenol	125	16.0	27.2	5.75	4.8	7.1
4.	Propiconazole	125	12.4	19.6	6.07	2.5	4.8
5.	Prochloraz	400	19.4	24.9	5.89	2.6	3.5
6.	Prochloraz + carbendazim	400 + 150	7.7	14.4	6.08	1.2	2.0
7.	Propiconazole + carbendazim	125 + 100	6.8	14.4	6.00	3.2	4.9
8.	Triadimenol + tridemorph	125 + 375	14.6	29.4	5.90	3.7	6.9
9.	Propiconazole + tridemorph	125 + 250	8.4	14.7	6.12	1.7	3.5
10.	Propiconazole (2 sprays)	125	2.8	8.2	6.76	0.6	3.3
11.	Flusilazole + carbendazim	156 + 78		Not Tested		0.8	1.8
SED			2.22	3.70	0.17	0.67	0.92
DF			81	81	54	60	60

a. Applied as a single spray at GS 31, except for Treatment 10 (sprayed at GS 31 and GS 39)

b. Plot yields were not determined for 1989 trials.

DISCUSSION

In his 1981 survey of sensitivity to triadimenol in R. secalis, Hollomon (1984) found only one out of 30 isolates with an MIC above 1.6 mg/l, and that isolate had an MIC of 3.2 mg/l. In contrast, in 1987 46% of the isolates had an MIC of 6.4 mg/l or above, so there had clearly been a shift in the pathogen population towards decreased sensitivity to triadimenol. By 1989 the population had become less sensitive than in 1987, and MIC values higher than 12.8 mg/l triadimenol were recorded. The shift between 1987 and 1989 was even more marked with propiconazole; the results for the 2 years cannot be compared over the whole range of concentrations, but the concentration of 0.2 mg/l was used in both years and the increase from 5% to 70% in the proportion of isolates with an MIC of 0.2 mg/l or higher shows how the population changed. There is no directly comparable early 1980s data for propiconazole. The bimodal distribution of triadimenol sensitivity in R. secalis is in contrast with the sensitivity of wheat and barley mildew (Erysiphe graminis f.sp. tritici and E. graminis f.sp. hordei) to DMI fungicides, which shows a unimodal distribution (Schulz & Scheinpflug, 1986; Wolfe, 1985). In view of the propensity of other pathogens to develop resistance to carbendazim it is surprising that R. secalis has not become resistant. The reason for this may be, at least in part, that carbendazim-generating fungicides have often been used on barley in mixtures with other fungicides which also have activity against R. secalis.

The field trials showed that propiconazole and prochloraz gave reasonable control of leaf blotch, but that disease control was improved by the addition of carbendazim or tridemorph, particularly in 1988. Flusilazole plus carbendazim was the most effective treatment in 1989, but flusilazole was not included alone. Triadimenol was less effective than the other DMI fungicides in the field, consistent with the finding in vitro that at any given dose propiconazole is more active than triadimenol against R. secalis. The pathogen populations at the 1989 sites were more sensitive to DMI fungicides than those at the 1988 sites, which may in part account for the better performance of DMI fungicides in 1989 than 1988. However, it is also possible that efficacy of sprays was affected by the timing of sprays in relation to when infection occurred, in that fungicides may differ in eradicant or protectant activity.

To demonstrate that resistance to DMI fungicides has developed in practice in R. secalis critical data is required to show that the performance has declined. It is not yet certain that R. secalis has become resistant in practice to DMI fungicides. At the present levels of sensitivity in R. secalis, the DMI fungicides, with the possible exception of triadimenol, are still effective for leaf blotch control. However, there is a danger that, if the population shifts further, disease control might be affected. It would be sensible to use DMI fungicides in mixtures with fungicides of different modes of action for leaf blotch control, because of the greater efficacy of the mixtures and the possibility that resistance is less likely to develop to DMI fungicides used in mixture than when used alone.

ACKNOWLEDGEMENTS

I am indebted to Miss K A Birks, Miss J C Purbrick and Mr R Marchant for technical assistance. This work was funded by the Home-Grown Cereals Authority.

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SENSITIVITY TO PROPICONAZOLE OF *RHYNCHOSPORIUM SECALIS* POPULATIONS FROM INDIVIDUAL BARLEY FIELDS IN NORTH EAST SCOTLAND AND EAST ANGLIA

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ABSTRACT

The sensitivity of populations of *Rhynchosporium secalis* to propiconazole from barley fields in north-east Scotland and East Anglia in 1989 was similar prior to and after a commercial fungicide programme based on propiconazole. EC50 values for 35 to 40 isolates from individual fields varied from <0.01 to >0.3 mg/l. The results indicated that no shifts in sensitivity occurred over the course of a season and that there was a similar sensitivity profile for a high disease pressure area (Scotland) as a low pressure area (East Anglia). The wide range in EC50 values at each site indicated the need to compare the sensitivity within populations of *R. secalis* from individual locations to decide whether sensitivity shifts have taken place.

Excellent disease control was obtained with the propiconazole programme in both areas. Tridemorph (350 g AI/ha) and carbendazim (100 g AI/ha) both contributed to disease control and in combination with propiconazole either of these partners should reduce the risk of resistance development.

INTRODUCTION

In wet seasons epidemics of leaf blotch, caused by *Rhynchosporium secalis*, can cause substantial losses in winter barley in the UK, particularly in south-west England, Wales, Northern Ireland and north-east Scotland. In trials in Scotland in 1988 the disease was responsible for yield reductions of up to 46% if an effective fungicide spray programme was not followed (Kirk and Leadbeater, 1990). In other parts of the U.K., the disease is relatively less important.

Fungicides which inhibit the C-14 demethylation step of sterol biosynthesis (DMI fungicides), especially those belonging to the triazole and imidazole chemical groups, have been the most widely used products to control the disease since the late 1970s. Efforts to monitor any sensitivity changes in *R. secalis* in response to the widespread use of DMI compounds on cereals have been made out throughout the 1980s. Significant variation, as measured by minimum inhibitory concentration (MIC), was found in the sensitivity to triadimenol of single spore isolates of *R. secalis* collected in 1981. Four isolates collected prior to the use of DMIs were not especially sensitive to triadimenol (Hollomon, 1984). Shifts in MIC from 0.3 mg/l to greater than 10 mg/l for triadimenol and 0.01 mg/l to 0.3 mg/l for prochloraz and propiconazole could be demonstrated after four successive transfers on greenhouse plants treated with these fungicides (Hunter *et al*, 1986). Nationwide surveys funded by the Home Grown Cereals Authority conducted between 1987 and 1989 have established MIC values for triadimenol for a large number of isolates (Hollomon, pers. comm.). Comparing these figures for triadimenol with the limited numbers obtained in

1981, the study concluded that sensitivity to DMI fungicides has declined in the U.K. (Anon 1989). Very little, however, has been published on the results of surveys to monitor the sensitivity of *R. secalis* to other DMI fungicides.

The objective of the present study was to determine the range in sensitivity to propiconazole of populations from individual barley fields before and after a commercial spray programme in 1989. In the absence of base-line sensitivity data to propiconazole prior to its introduction, a comparison of the sensitivity of populations from a high disease pressure area was made with a low pressure one. In the two Scottish fields where sampling was undertaken, small plot replicated trials were also carried out to determine the performance of the component parts of propiconazole mixtures containing carbendazim and tridemorph.

MATERIALS AND METHODS

Monitoring field populations

Two high pressure disease sites were located in the Aberdeenshire area of Scotland where leaf blotch is a serious disease problem in most years. The low pressure site was situated in East Anglia, an area where leaf blotch is usually of minor importance. Sampling was carried out over an area of about 1 ha in a 'W' transect with 40 sampling stations immediately prior to and three weeks after the completion of a fungicide programme for each site. The cultivar on both Scottish sites was Magie and on the east Anglian site was Plaisant. The sampling area on all sites received a typical commercial programme for the region. In Scotland this was propiconazole + fenpropimorph (125 + 750 g AI/ha) at the first application (GS 31) and propiconazole + tridemorph (125 + 250 g AI/ha) at the second application (GS 39). At the East Anglian site two sprays of propiconazole + tridemorph (125 + 350 g AI/ha) were applied at GS 31 and 39.

Leaf pieces with individual leaf blotch lesions were cut into 3 x 5 mm portions and immersed in 70% ethanol for two minutes. After rinsing in sterile water and blotting dry, they were placed on Difco yeast malt agar (YMA) amended with streptomycin sulphate. Petri dishes were incubated at 21°C in the dark for 7-10 days after which the fungus was streaked onto fresh YMA and incubated for 7 days. Plates were then flooded with sterile distilled water and scraped with a scalpel to remove the spores. The concentration of the resulting suspension was then adjusted to 1×10^6 - 1.5×10^6 spores/ml. The effective concentration for inhibition of 50% of the yeast-like colony growth (EC50) was then determined for one isolate from each sampling station on YMA agar amended with 0, 0.01, 0.1, 0.5, 1.0 and 10 mg/l propiconazole. Three replicate 50 mm plates were inoculated with 300 µl of spore suspension distributed uniformly over the surface. Growth was assessed for percent surface area colonised and plotted on semi-logarithmic paper.

Two reference isolates known to differ in sensitivity to triadimenol, RSACP and S238.02, were supplied by Long Ashton Research Station and used with every test to ensure uniformity of results.

Field trials

Small plot trials in Scotland consisted of plots 2.5 x 8 metres which were replicated four times. Fungicides were applied using a knapsack sprayer with side-held boom and a water volume of 200 l/ha. Treatments to small plot trials were applied on the same date as treatments to the large sampling area. Leaf blotch was assessed as the percentage leaf area affected on the top three leaves at the time of the first application and at regular intervals thereafter. On the East Anglian site there was no small plot trial, but within the field there was an untreated area in which the disease progress was monitored.

RESULTS

Sensitivity tests

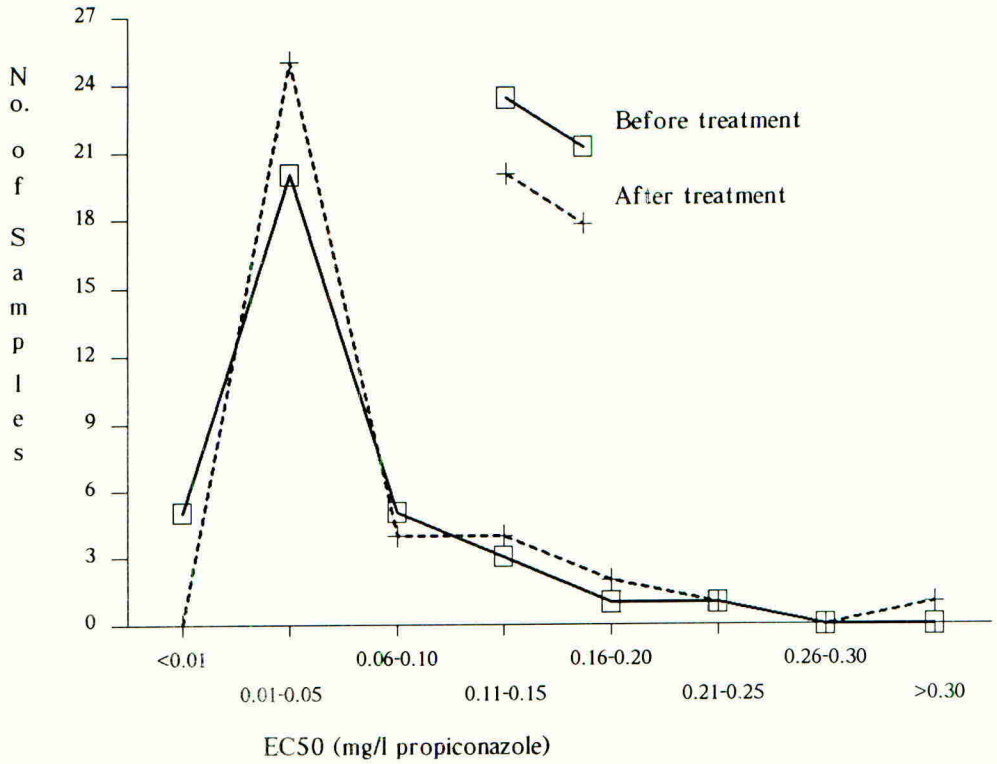
The EC50 values for the reference isolates expressed as a mean of 10 *in vitro* tests were 0.03 mg/l for isolate RSACP and 0.20 mg/l for isolate RS238.02.

EC50 values were obtained for 35 - 40 isolates from one Scottish site and the East Anglian site prior to and after fungicide application. Isolations from site 2 in Scotland were mostly unsuccessful due to contaminants. There was a wide range of EC50 values, from 0.005 to 0.35 mg/l, but at all sites isolates were predominantly in the range 0.01 to 0.05 mg/l irrespective of location or whether sampled before or after fungicide treatment. The sensitivity spectrum for the Scottish site 1 and the E. Anglian site is shown in table 1 and in figures 1 and 2 where the number of isolates in different EC50 category groups are plotted prior to and after fungicide treatment.

Table 1. Sensitivity of *R. secalis* from winter barley fields before and after fungicide treatment in 1989.

EC50 mg/l propiconazole	Scotland Site 1		East Anglia	
	before	after	before	after
0.005 - 0.01	5	0	7	0
0.01 - 0.05	20	25	22	26
0.06 - 0.10	5	4	4	11
0.11 - 0.15	3	4	0	2
0.16 - 0.20	1	2	2	1
0.21 - 0.25	1	1	0	0
0.26 - 0.30	0	0	0	0
0.30 - 0.35	0	1	0	0
Total	35	37	35	40

FIGURE 1. Sensitivity of *R. secalis* to propiconazole in 1989 before and after fungicide treatment: **Scotland Site 1**



Fungicide performance

In East Anglia, leaf blotch was the only disease present and built-up to 15% foliar attack in the untreated area soon after the second fungicide application. In the treated area less than 1% leaf blotch attack occurred.

In Scotland moderate levels (25 - 30%) of leaf blotch attack occurred in untreated plots in both trials which were well controlled by a two spray programme of propiconazole (table 2). Tridemorph applied at 350 g AI/ha gave 40% and 51% control of leaf blotch and carbendazim gave 52% and 35% control respectively in the two trials. The combination of either of these products with propiconazole did not result in an improved level of control over that of propiconazole alone. Brown rust occurred in both trials, reaching 52% foliar attack at site 1 and 16% at site 2 in the untreated plots. This was controlled by treatments containing propiconazole but not by carbendazim and only poorly by tridemorph. Combinations of propiconazole with tridemorph on both sites and with carbendazim on one site showed a trend towards higher yields compared with propiconazole alone but differences were not statistically significant.

FIGURE 2. Sensitivity of *R. secalis* to propiconazole in 1989 before and after fungicide treatment: E. Anglia

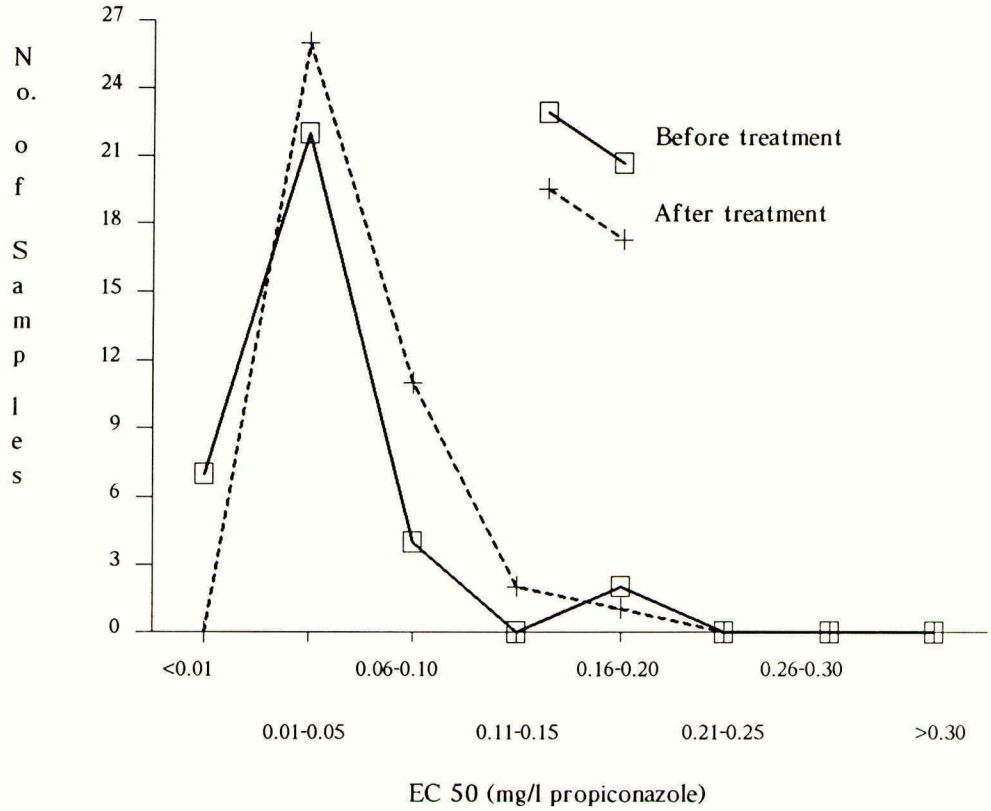


TABLE 2. Control of leaf blotch with fungicides on two Scottish sites in 1989

Treatment	Rate g AI/ha	% leaf blotch 27 DAT* (GS75)		Yield as % of untreated (16% moisture)	
		Site 1	Site 2	Site 1	Site 2
untreated	-	25.0	31.3	4.2 t/ha	6.0 t/ha
propiconazole	125	2.8	3.5	125	135
tridemorph	350	10.0	16.0	115	113
carbendazim	100	13.0	11.0	101	90
propiconazole + tridemorph	125 + 350	3.0	2.8	131	141
propiconazole + carbendazim	125 + 100	6.8	4.5	147	127
Tukey LSD 5%				32	24

* Days after the GS 39 treatment
Application dates: 1. 7/3/89 (GS 31); 2. 5/5/89 (GS 39).

DISCUSSION

Favourable climatic conditions for leaf blotch development and the intensive use of fungicides make north east Scotland a likely area for the development of shifts in sensitivity to fungicides. The results of the intensive monitoring that was carried out in 1989 showed that there were no differences between the sensitivity of populations of *R. secalis* to propiconazole between this area and a low disease pressure area in East Anglia. This would suggest that the risk for major sensitivity shifts to propiconazole under field conditions in the UK is relatively low. This is further supported by the lack of change in the sensitivity of populations in both areas over a single season following a programme based on propiconazole.

Any substantial shifts in sensitivity would be reflected in fungicide performance. Trial results over the past 3 seasons have shown that propiconazole gives consistently high levels of disease control (Kirk and Leadbeater, 1990). If shifts to propiconazole have occurred since its introduction then they lie within the range where the fungicide is efficacious. Propiconazole can therefore be considered as having a greater 'reserve' than some of the weaker DMI fungicides. This is supported by studies carried out by Girling *et al* (1988) which showed that in a DMI resistant strain of *R. secalis* there was a lower level of resistance to propiconazole than to triadimenol. Propiconazole inhibited the C-14 demethylation step as well as affecting sterol biosynthesis beyond this step. This field and laboratory study has shown that there is a wide range in the sensitivity of *R. secalis* within individual sites and that it is necessary to determine the comparative range in sensitivity of populations in deciding whether sensitivity shifts have taken place. It is our intention to carry on monitoring the sensitivity of populations on the Scottish sites especially in years in which the disease pressure is high. The data obtained in 1989 will then serve as a reference for identifying any future shifts which might occur.

Propiconazole used alone gave excellent disease control on all three sites and both tridemorph and carbendazim applied at rates below those recommended contributed to disease control. The use of mixtures of fungicides having a different mode of action provides a valuable strategy to hinder the development of resistance in *R. secalis* and should also be advocated for the broadening of the spectrum of diseases controlled.

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METHODS TO TEST THE SENSITIVITY OF MYCOSPHAERELLA FIJIENSIS TO TRIADIMENOL

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ABSTRACT

Methods are presented which can be used to differentiate between Mycosphaerella populations in bananas with respect to their sensitivity to azole fungicides. A germ tube elongation test gave a general overview of a population as a whole. Detailed information was obtained with the aid of a whole plant test, where small subpopulations of the pathogen were used. Benefits of the respective methods are discussed, as well as their particular advantages for specific research topics.

INTRODUCTION

The control of the Sigatoka diseases (M. fijiensis, M. musicola) is achieved primarily through the use of fungicides, since resistant banana varieties are presently not available. Dithiocarbamates and benzimidazole fungicides have been the most frequently used classes of chemicals, although in recent years they have been increasingly replaced or supplemented by azole fungicides.

It has been shown that the continuous use of benzimidazoles can lead to the selection of resistance within Mycosphaerella populations (Anonymous, 1986). This process is thought to have contributed to the loss of control of M. fijiensis under field conditions. Test methods have been developed to determine and monitor the development of resistance to fungicides. Various test procedures have been reported by researchers such as Fouré et Mouliom Pefoura (1988), Mourichon et al. (1987), Shillingford (1984), Pasberg-Gauhl (1989) and Anonymous (1986).

Experience in other crops has also shown that the intensive use of azole fungicides may sometimes lead to the selection of less sensitive pathogen populations, but this is not a must.
In this paper, test methods are described which can be used to determine differences in the sensitivity of Mycosphaerella isolates to azole fungicides such as triadimenol.

METHODS

Germ tube elongation test

a: Petri-plate preparation

An agar-plate concentration series was prepared with triadimenol (10,3,1,0.3,0.1 µg AI/ml) by adding the active ingredient, dissolved in a solvent of 1 ml acetone filled up to 100 ml with water, to a 2% water-agar solution, which was then poured into 9 cm Petri-plates. The control plate contained only the solvent/agarsolution mixture.

b. Test procedure

Banana leaves which contained ripe perithecia were required for the test. Sections (4x4 cm) were cut from infected leaves and immersed in water for 10 minutes. Each leaf section was attached to a moistened filter-paper disc which was then placed in a petri-plate cover. The moistened filter paper adhered to the plastic cover, which was then placed over a petri-plate containing agar. In this way each petri-plate in the test series contained a leaf sample from the test source. The position of the leaf section above the agar was outlined on the bottom of the petri-plate, using a marking pen.

The leaf sections remained suspended over the agar plates for 1 hour, during which time the ascospores were released. Following the removal of the leaf sections, the petri-plates were recovered, stored in closed transparant plastic containers, and placed in a growth chamber for 32 hours under controlled conditions (26°C and illumination).

c. Evaluation

The spores on the agar-plates were killed with a solution of 3 parts formaldehyde (38%) + 1 part water. Each petri-plate was first observed for spores under a low-power binocular with translucent lightning. Detailed evaluations were then carried out under a microscope (200x; objective 20x / ocular 10x with ocular-micrometer). Generally, 50 randomly selected spores were evaluated per petri-plate, starting with the control plate.

With the aid of the ocular-micrometer the length of the germinated, two-celled spore was measured. The average of 50 germ tube lengths was calculated (excluding the spore length) and then halved to give a "TS/2" value. This value served as a standard with which the data from the concentration series were compared.

For each fungicide concentration the numbers of spores whose germ tube length was $>$ or $\leq$ TS/2 (half the control) were recorded. The Abbott values (effectiveness) of each concentration, after probit-transformation, were correlated to the logarithms of the concentrations using a weighted probit-analysis. ED 50 and ED 95 values for each sample were calculated.

Test with whole plants

a. Production of mycelia

Ascospores were produced using the methods described for the germ tube elongation test. Using a low power binocular microscope with translucent lightning, small groups of spores were isolated, transferred with the aid of an insect needle to Mycophil agar (15g agar + 10g dextrose + 10g soyapeptone + 1l distilled water), and cultured under standard conditions (26°C/light). After 1 to 2 weeks, depending upon the speed of growth, the cultures were streaked out and cultured further.

Later, small pieces of mycelium were transferred to liquid medium (30 ml of a 10g dextrose + 10g soyapeptone + 1l distilled water solution contained in a 100 ml Erlenmeyer flask). The cultures were incubated without shaking them for 2 weeks (26°C/light), during which time numerous mycelium flakes were formed.

b. Plants

Banana plants, var. Grand Naine of the Cavendish Group, grown from cell cultures (produced by VITROPIC, Saint-Mathieu-De-Tréviers, France) were used in the tests. The 3-4 cm sized plants were delivered growing in petri plates containing a sterilized medium. The plants were transplanted into small pots containing potting soil and grown at 26°C and 80% r.h. under light. The plants were later transferred to 14 cm diameter pots. Plants which had reached a height of approximately 20 cm and 7 leaves (approximately 40 days after receipt) were used in the test.

c. Test methods

I. One day prior to inoculation, triadimenol was applied (as '(R)Bayfidan 1 GR' granules) to the potting soil at rates of 30,10,3,1 or 0.3 mg AI/pot. Each pot was drenched with 100 ml of water. The control received only water.

II. One day prior to inoculation, 20 ml of a solution containing triadimenol (as '(R)Bayfidan 250 EC') was added to the test pots at rates of 30,10,3,1, or 0.3 mg AI/pot. The control received only water + blank formulation. Each pot was drenched with an additional 20 ml of water.

III. Shortly before inoculation, plants were sprayed with 50 ml of an aqueous triadimenol solution (as '(R)Bayfidan 250 EC') containing 300,30,3,0.3 µg AI/ml water. The control was sprayed with water + blank formulation. The plants were allowed to dry for 1 hour prior to inoculation.

Following the various treatments, the plants were inoculated by removing, with the aid of forceps, 10 mycelium flakes from the liquid culture and placing them in two rows on the underside of the youngest newly unrolled leaf.

The inoculated leaf of each plant was enclosed for 10 days in a plastic bag in order to promote infection. During the trial the plants were maintained under the standard conditions described previously.

d. Evaluation

The trial was evaluated 4 weeks following inoculation. The radial expansion of the fungus (diameter) within the leaf was measured on the upper leaf surface and the 10 values per leaf were averaged to provide a "test unit". The Abbott values (% activity) for the individual treatment concentrations were determined and a weighted dose-response-analysis was calculated. The relative sensitivity of each fungal isolate was characterized with the aid of ED 50-and ED 95 values.

RESULTS AND DISCUSSION

This study confirms and partly modifies suggestions reported in the literature. The results demonstrate that the germ tube elongation test and the test with whole plants are very suitable for determining the sensitivity of Mycosphaerella isolates.

Several conditions must, however, be met. The leaf samples collected should show advanced symptoms of the disease and must contain fruiting bodies. The sampling must be carefully carried out, especially when the tests cannot be run at the site of the banana plantation and new samples cannot be readily obtained, when no ascospores are produced.

In contrast, conidia cannot be collected from detached leaf samples if these must be packed and shipped.

Care should be taken not to evaluate the spore germ tube elongation test too early. Since azole fungicides do not inhibit spore germination, it is possible that unspecified factors may exert a greater influence on the germ tube growth in a short-term study than does the actual inhibition of sterol biosynthesis. Tests of different duration showed that 32 hours was a suitable time period (Table 1). For other fungi, measurable effects on the germ tube elongation after inhibition of sterol biosynthesis are often not detectable during the first 24 hours (R. Pontzen, BAYER AG, pers. comm.). Evaluations after 48 hours showed similar results to those obtained after 32 hours, but had the additional disadvantage that the evaluation may have been adversely influenced by the growth of other fungi or bacteria. Examples of typical germ tube elongation tests are shown in Table 2.

Attempts were made to obtain exact germtube measurements for each test concentration in order to calculate an exact dose-response relationship. However, this proved to be too time consuming and is therefore much less suitable for routine studies than the counting method. The $\leq$ 50% size limit was selected based on our own requirements and test considerations, but other limits could also be selected.

M. fijiensis, as well as other species in the genus, loses its pathogenicity very rapidly when maintained in culture. It is therefore essential to propagate the culture as rapidly as possible using the minimum steps of subculture. This problem results in the fact that it is hardly possible to include a standard fungus isolate as an internal standard, unless it can be cultivated on intact plants. The time and effort required for growing and maintaining the crops and standard fungi is very high, expecially for tests which are performed only occasionally. Field samples from treated plants should therefore always be compared directly with samples taken from untreated plants.

The age of the plants in the test appeared to be of much less importance than the age of the leaves selected. Newly unrolled leaves and those from the next leaf level, appeared best suited for rapid infection (Table 4). All the three application types used for the whole plant test were easy to conduct, but method II (soil drench) proved to be the most suitable one (Table 5).

The advantage of this type of plant test lies in the very simple and rapid evaluation method which is comparable to a radial mycelium growth test conducted on agar plates. Since the necrosis does not always develop in a perfect circle, it is suggested that the average of two separate diameter measurements in two directions should be determined.

The test procedure, from the isolation of the fungus until the final evaluation, is relatively lengthy, but clearly shows differences in sensitivity, for example between isolates 89/17 and 89/19 (Table 3). Thus the

test is well suited for determining differences in sensitivity among subpopulations but is too time consuming for rapid, routine studies.

On the other hand, experience with other pathogens has shown that only detailed studies can provide accurate information on the variability of the sensitivity values within a given basic population. Only then it is possible to make an early identification of small changes or shifts in the sensitivity of the pathogen population to azole fungicides. A less detailed test provides a general overview of the situation but often cannot identify small changes or differences (compare Tables 2 and 3). It is therefore useful to have both methods available.

Further studies must be conducted to determine whether or not this test method can be used to study M. musicola. The ascospores from M. musicola do not produce germtubes as straight as those of M. fijiensis and the germ tubes branch rapidly. Therefore an evaluation scale other than the length measurement must be used in the germ tube elongation test. The plant test should be able to be readily used for both species. However, it may be necessary to select lower test dose rates since M. musicola generally reacts more sensitively to triadimenol than does M. fijiensis.

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TABLE 1. Ascospores: Germ tube elongation test on sample 89/6; different time periods in test

µg triadimenol/ml	test 1		test 2
	24 h	48 h	32 h
30	26 *	100	100
10	12	100	100
3	60	96	98
1	24	24	46
0,3	2	12	2
ED 50	--	1,14	1,05
ED 95	--	4,46	3,17

* Abbott values

TABLE 2. Ascospores: Germ tube elongation tests on samples 89/17 and 89/19

ED values	test 2	test 1	
	89/17	89/17	89/19
ED 50	1,149	0,533	0,733
ED 95	2,951	2,410	2,312

TABLE 3. Mycelium: Test on whole plants with triadimenol (method II)

ED values	isolates	
	89/17-2	89/19-2
ED 50	12,514	2,089
ED 95	18,909	3,679

TABLE 4. Mycelium: Test on whole plants with triadimenol (method III) isolate: 90/5-1/2

µg triadimenol/ml	just unrolled leaf	first leaf	both leaves
300	100	100	100
30	79,7	89,9	84,7
3	27,0	21,7	25,0
0,3	0	11,6	0
ED 50	7,932	7,027	7,382
ED 95	124,278	51,673	79,532

TABLE 5. Mycelium: Test on whole plants - different methods isolate 89/6-8 (shortened concentration series)

Method	ED 50-values for triadimenol	
	mg/pot	µg/ml
I Granules	3,130	--
II Soil drench	0,548	--
III Spray	--	34,465

LONG-TERM MONITORING OF PSEUDOCERCOSPORELLA HERPOTRICHOIDES POPULATIONS FOR SENSITIVITY TO PROCHLORAZ AND CARBENDAZIM

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ABSTRACT

Populations of Pseudocercosporella herpotrichoides were monitored for sensitivity to prochloraz and carbendazim from 1985 to 1989. The pathogen was isolated from cereal stems obtained from field trials in France, West Germany the UK and Denmark, and in vitro testing carried out on agar amended with the fungicides. The reaction to prochloraz has remained largely unaltered throughout the survey and is similar to that reported by other workers for isolates obtained in 1984. The proportions of W and R types isolated were different in the four countries. The R type was relatively more frequent than the W in the UK and Denmark, with the opposite being true of France and Germany. However, all of the countries have seen a decline in the percentages of R types isolated over the period of this survey. Carbendazim resistance increased in all countries, and in both the W and R type sections of the population. The frequency of resistance was much higher in the UK and France than in West Germany and Denmark.

INTRODUCTION

Eyespot of wheat and barley, caused by Pseudocercosporella herpotrichoides, is a major cause of yield and quality loss wherever autumn-sown cereals are grown intensively. Carbendazim has been used to control eyespot for a number of years but resistance to this fungicide is now widespread in the UK (Hollins and Scott, 1987), France (Cavelier et al, 1985), West Germany (Schreiber and Schlesinger, 1985) and Denmark (Nielsen and Schulz, 1985).

The pathogen population can be divided according to growth in vitro into two morphological types. One is fast-growing and has a smooth-edged, domed appearance on potato dextrose agar (PDA), while the other is slower growing and has a feathery-edged appearance. These two types are usually refered to as the wheat (W) and rye (R) types respectively (Hollins et al, 1985), and have been found to vary considerably in their reaction to fungicides such as the triazole group of DMIs. However, prochloraz has been found to be equally effective against all components of the pathogen population (Leroux and Gredt, 1985).

Since 1983, Schering has been monitoring populations of P. herpotrichoides derived from field trials in France, Germany, Denmark and the UK.

The in vitro responses of isolates to prochloraz and carbendazim were evaluated and the morphological types determined. The results of work with isolates obtained prior to and including 1984 were reported by Gallimore et al. (1987). Birchmore et al. (1986) reported on isolates from 1985 cereal crops. This paper presents the data from 1985 to 1989.

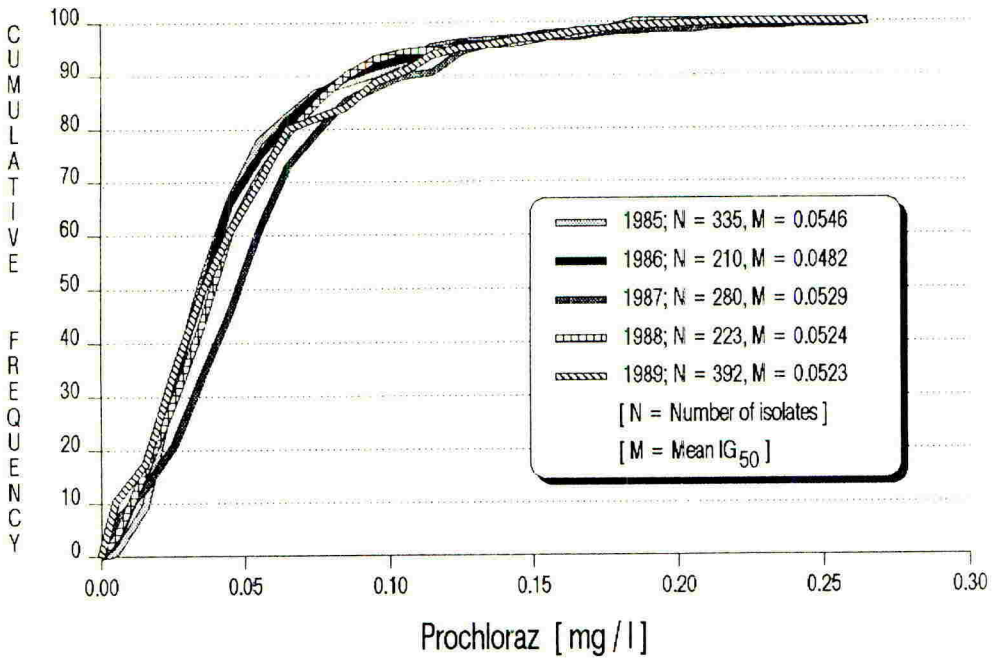
MATERIALS AND METHODS

The method of isolation of P. herpotrichoides from eyespot lesions was as reported by Birchmore et al. (1986) for in vitro evaluation of sensitivity to fungicides. Plugs of agar (3mm diameter) were taken from the edge of each isolate and placed, mycelium downwards, onto petri dishes containing PDA amended with a range of concentrations of prochloraz (0.0050, 0.0075, 0.010, 0.025, 0.050, 0.075, 0.10, 0.25 and 0.50 mg/l) or 2.0 mg/l carbendazim. Colony diameters were measured after a further 14 days growth at 20°C and the prochloraz results for each isolate subjected to linear regression against the logarithm of the prochloraz concentration in order to calculate the IG_{50} value, that is, the concentration required to give 50% inhibition of growth. The isolates were also characterised as either W or R types of the pathogen.

RESULTS AND DISCUSSION

A total of approximately 6500 isolates of P. herpotrichoides from the four countries included in the survey have been examined since 1985.

FIGURE 1. Distribution of IG_{50} values, 1985 to 1989

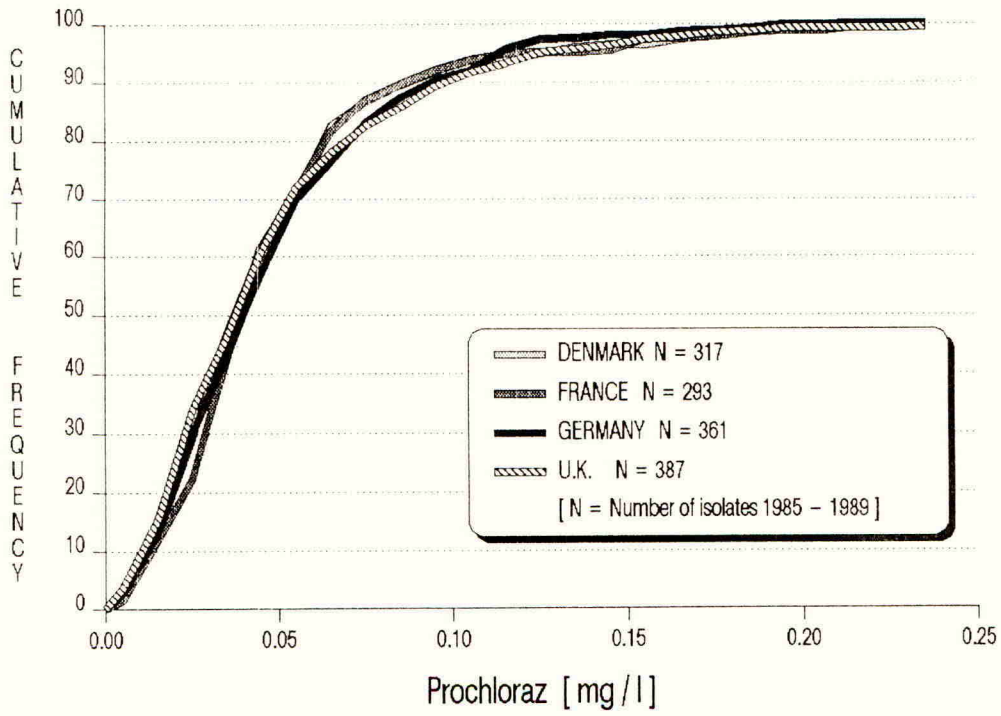


All of the isolates were tested for resistance to carbendazim, while a random selection were included in the prochloraz dose-response test. The results of the latter are shown in Figure 1.

The cumulative frequencies of the IG_{50} values obtained each year from 1985 to 1989 are shown, together with the numbers of isolates investigated (N) and the mean IG_{50} values for each year (M).

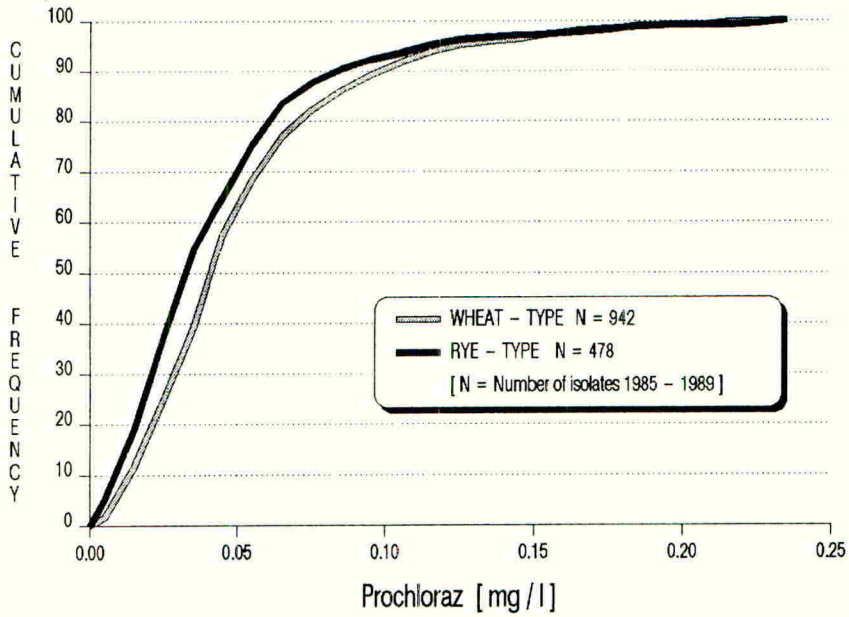
The similarity of the cumulative frequency curves indicate that the response to prochloraz has remained unchanged over this period and this is confirmed by the mean IG_{50} values. The latter are similar to the value of 0.044 ± 0.01 obtained in 1984 by Gallimore et al. (1986). The mean for 1985 isolates of 0.0546 mg/l prochloraz is higher than the 0.036 mg/l quoted for that year by Birchmore et al. (1986) but is based on evaluation of 335 isolates rather than the 224 for which data were available at that time. The reaction of isolates from the four countries included in the survey has remained similar (Figure 2).

FIGURE 2. Distribution of IG_{50} values of isolates from the UK, France, West Germany and Denmark



The cumulative frequencies of the IG_{50}s of W and R type (Figure 3) show that the latter has been consistently, although only slightly, more sensitive to prochloraz than the W type. This agrees with the findings of both Gallimore et al. (1987) and Birchmore et al. (1986).

FIGURE 3. Distribution of IG_{50} values of W and R types



The proportions of W and R types isolated during these years are shown in Table 1.

TABLE 1. Percentages of isolates classified as W or R morphological types

	UK		FRANCE		GERMANY		DENMARK	
	W	R	W	R	W	R	W	R
1985	19.0	81.0	54.9	45.1	66.8	33.2	22.2	77.9
1986	26.7	73.3	79.7	20.3	67.1	32.9	55.4	44.6
1987	45.5	54.5	73.3	26.7	83.7	16.3	61.2	38.8
1988	64.5	35.5	77.0	23.0	79.0	21.0	19.0	81.0
1989	58.0	42.0	71.7	28.3	56.9	43.1	59.0	41.0

In the past the R type was much less common that the W and there are reports, prior to the 1980s, of it comprising only a relatively low percentage of the population (Hollins et al., 1985). The proportion of R types then rose during the early 1980s, (Hollins and Scott, 1987) attributed in part to selection pressure from the carbendazim generating fungicides (Hoare et al., 1986).

At that time these were more effective against the W type than the R type. However, in our survey the percentages of R types in samples from all four countries have fallen since the middle of the decade. This trend was most marked in the UK, where in 1985 the percentage of R types was at a peak of 81%, while by 1988 this was reduced to 35%.

In France the R type has always been in the minority, 45% of isolates were in this category in 1985 but by 1986 this had fallen to 20.3% and has only increased slightly every year since then to 28.3% in 1989.

In Germany the proportion of R types fell dramatically, from 66.3% in 1984 to 33% in 1985, and to 16.3% in 1987. Since then there has been a recovery to 43% in 1989.

The Danish population appeared to be much more variable, initially resembling that of the UK with 78% of the population being R type in 1985, falling to 38.8% in 1987. The peak of 81.0% R types in 1988 is probably an anomaly due to the very small number of 52 isolates recovered from Danish samples during that year.

The fluctuations in the frequencies of the two types may be due to climatic factors, such as rainfall and the unusually high winter temperatures of the last two years. Alternatively, changes in agronomic practices such as the date of sowing winter cereals, the proportion of wheat/barley crops or changes in varieties may play a part in these fluctuations. In addition, the use of azole fungicides for the control of cereal foliar pathogens may have an effect on the eyespot population. Many of these compounds are not sufficiently active against P. herpotrichoides to be recommended for eyespot control but are known from in vitro work to have an effect on the pathogen. For instance, triazole fungicides are known to be active against the W, but not the R type (Leroux and Gredt, 1985) and this could exert a selection pressure on the population.

The percentage of isolates resistant to carbendazim and the proportions of the W and R type populations with this property are shown in Table 2.

TABLE 2. Percentages of W and R type isolates from 4 countries resistant to carbendazim, 1985-1989

	UK		FRANCE		GERMANY		DENMARK	
	W	R	W	R	W	R	W	R
1985	28.1	83.5	82.6	95.1	27.3	4.9	11.1	72.6
1986	51.0	83.6	72.6	100.0	46.4	68.0	47.6	71.8
1987	75.6	90.7	53.4	100.0	31.7	60.0	27.0	43.8
1988	69.6	65.8	77.0	89.0	23.0	38.0	10.0	24.0
1989	70.5	91.2	89.7	96.4	60.6	51.0	17.0	44.0

The percentage of isolates resistant to carbendazim was very high in both the UK and France, but was much lower in Germany and Denmark.

Overall, resistance to carbendazim is much more common in the R types than in the W types, although in some years, particularly in the UK (1988) and Germany (1985 and 1989) the converse occurred. Levels of carbendazim resistance in the R type are extremely high in both France and the UK.

The underlying trend in all countries, except Denmark, was for carbendazim resistance to increase in both the W and the R types.

CONCLUSIONS

In conclusion, the reactions to prochloraz of populations of P. herpotrichoides from the UK, France, West Germany and Denmark have remained stable over the period of this survey. The R type of the pathogen appears to be consistently more sensitive to prochloraz than the W type, although the difference is relatively small.

This stability of the reaction of the pathogen to prochloraz is in sharp contrast to the marked changes in the proportions of the morphological types and the increase in resistance to carbendazim detected in the survey. These illustrate the capacity for change inherent in the eyespot population and emphasize the need for continued international monitoring.

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SENSITIVITY OF BARLEY POWDERY MILDEW ISOLATES TO MORPHOLINE FUNGICIDES

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ABSTRACT

The sensitivities of isolates of barley powdery mildew, *Erysiphe graminis* f. sp. *hordei*, collected during 1988-1990, to tridemorph, fenpropimorph and fenpropidin were determined (EC_{50} and MIC) in tests with detached leaf segments. The pathogen showed a 12-fold range of sensitivity to tridemorph, a 10-fold range to fenpropimorph and a 44-fold range to fenpropidin. The patterns of sensitivity of the isolates to the three fungicides differed.

INTRODUCTION

Barley powdery mildew *Erysiphe graminis* f. sp. *hordei* is the major disease in barley in the United Kingdom (Holloman, 1980). The two main methods of mildew control are the use of resistant varieties and the use of translocated fungicides. Extensive use of some triazole fungicides has led to a decline in field performance associated with a decrease of sensitivity in the pathogen (Fletcher & Wolfe, 1981; Wolfe, 1985). Resistance of *Erysiphe graminis* f. sp. *hordei* to the triazoles is not uniform across Great Britain but reflects the intensity of fungicide usage in different areas. In Scotland morpholines have replaced triazoles as the main type of fungicide used in powdery mildew control (Gilmour, 1984). In the present study powdery mildew isolates from south-east Scotland were screened for their sensitivity to morpholines.

MATERIALS AND METHODS

Leaves infected with powdery mildew were collected from barley crops and trial plots in south-east Scotland during 1988-1990. The isolates were cultured on detached leaf segments of barley cv. Golden Promise, maintained on Davis minimal medium containing 80 mg/litre benzimadazole. Three fungicides were used at five concentrations, based on the normally recommended field spray (C): tridemorph (C=0.7 litre/ha 'Calixin' in 200 litres water/ha); fenpropimorph (C=1 litre/ha 'Mistral' in 200 litres water/ha); fenpropidin (C=1 litre/ha 'Patrol' in 200 litres water/ha). For tridemorph 1/32C, 1/16C, 1/8C, 1/4C and 1/2C were used giving concentrations of 0.082, 0.164, 0.328, 0.656 and 1.312 g AI per litre. In early experiments fenpropidin and fenpropimorph were used at concentrations of 0.117, 0.234, 0.469, 0.938 and 1.976 g AI/litre. In later experiments these concentrations were reduced because of the sensitivity of the screened isolates to the fungicides. The amended concentration range was 1/256C, 1/128C, 1/64C, 1/32C, 1/16C giving 0.015, 0.029, 0.058, 0.117 and 0.234 g AI/litre. Control plants were sprayed with water.

For each test, seedlings of barley cv. Golden Promise were grown, five to a pot, in a Burkhart Isolation Propagator. Before the ligules were visible on the second leaves (approximately 14 days old), the fungicide solutions were applied to seedlings in two pots in each of a pair of spray cabinets using a Humbrol spray gun: 5 second application time, followed by 15 minutes settling time. The chemicals were applied in ascending order of concentration. Treated sets of plants were kept apart for 24 hours before the preparation of the leaf segments and inoculation of the segments with isolates of powdery mildew. Thirty two replicate leaf segments were cut from the second leaves of the test plants from each concentration/spray cabinet combination. The leaf segments were placed on Davis minimal medium containing 80 mg/litre benzimadazole and then inoculated uniformly by tapping

heavily infected leaf segments covered with spores over the plates and distributing the spores with a fine paint brush. Four isolates were screened during each test. The leaf segments were incubated at 18°C in an illuminated incubator with a 12 hour light period in each day.

Leaf segments were assessed for percentage mildew cover 14 days after inoculation. Several tests were repeated three times to check reproducibility. The results were analysed with the aid of a Genstat 5 programme which fitted symmetrical logistic curves and calculated EC_{50} values (concentration of fungicide which reduced mildew cover to half that of the untreated control); MICs (the minimum concentration at which no visible growth occured) were also determined.

RESULTS

Figure 1 illustrates the sensitivity of forty-two barley powdery mildew isolates to tridemorph, expressed as EC_{50} values, in order of decreasing sensitivity. There was a 12-fold difference between the most sensitive and the least sensitive isolate. Several isolates showed a high sensitivity to tridemorph and the EC_{50} values fit a log-normal distribution. The MIC values for 95 isolates were determined (Figure 1: note that the x-axis is a logarithmic scale). The MICs show a log-normal distribution. Several isolates from pre-1988 were incorporated in the screen and had high sensitivity to tridemorph. Four isolates out of the 95 were able to grow on segments from plants sprayed with half the recommended field spray concentration.

FIGURE 1. Sensitivity of powdery mildew isolates to tridemorph

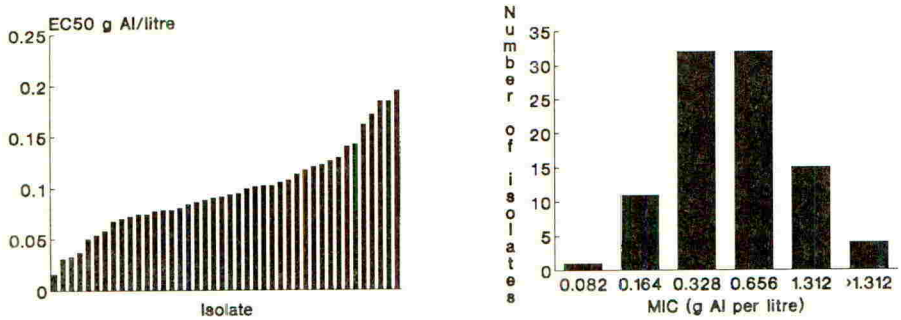


FIGURE 2. Sensitivity of powdery mildew isolates to fenpropimorph

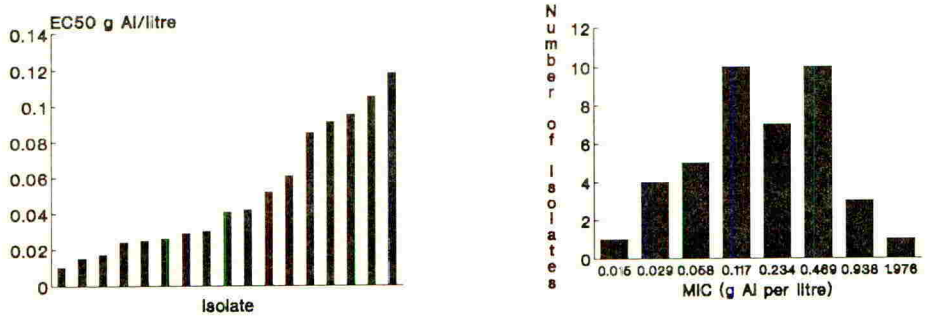
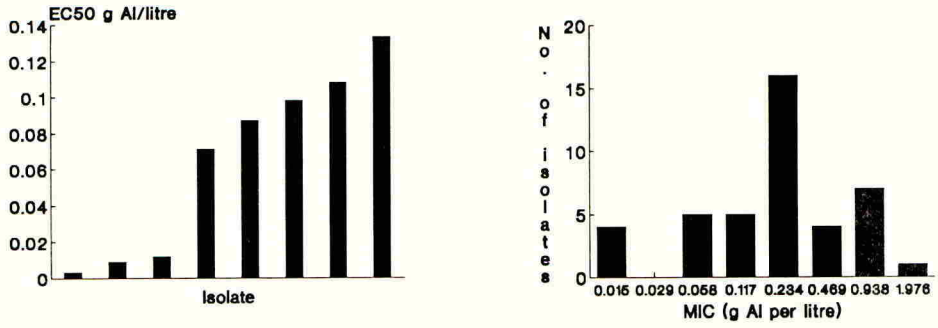


Figure 2 shows the sensitivity of isolates of powdery mildew to fenpropimorph, expressed as EC_{50} and MIC values. The EC_{50} values show a 10-fold range in sensitivity. The MICs show a heavy-tailed, left-skew distribution with several isolates showing high sensitivity to the fungicide.

The sensitivity of isolates to fenpropidin is illustrated in Figure 3. EC_{50} values have been calculated for fewer isolates but there was a 44-fold difference between the most sensitive and the least sensitive isolate. The MICs show a flat distribution with a slight left skew.

FIGURE 3 Sensitivity of powdery mildew isolates to fenpropidin



The patterns of sensitivity of isolates to the three fungicides differed (Table 1). For example isolate B35 had a relatively low sensitivity to tridemorph but was among the more sensitive to fenpropimorph and fenpropidin. PB had a lower sensitivity to fenpropidin but was more sensitive to fenpropimorph and tridemorph. BUSB18 was highly sensitive to tridemorph but showed a lower sensitivity to fenpropidin.

TABLE 1.Sensitivity of powdery mildew isolates to tridemorph, fenpropimorph and fenpropidin (EC_{50} in g AI/l)

Isolate	tridemorph	fenpropimorph	fenpropidin
R15	0.140	0.026	*
B35	0.122	0.024	0.012
P8	0.113	0.012	*
B21	0.101	0.105	*
MD	0.077	0.095	*
PB	0.070	0.061	0.108
B38	0.067	0.041	0.009
BUSB18	0.033	*	0.226
I9	0.031	*	0.071

DISCUSSION

The screened isolates of barley powdery mildew displayed a range of sensitivity reactions to the test fungicides. In general the isolates were more sensitive to fenpropimorph and fenpropidin than to tridemorph. This may be due to variation within the population, an effect of the distinct modes of action of the fungicides or a decrease in sensitivity to tridemorph. There was no evidence that reduced sensitivity to tridemorph was associated with lower sensitivity to either fenpropimorph or fenpropidin.

The isolates showed a log-normal distribution of sensitivity reactions to tridemorph. This distribution and the large difference in sensitivity to tridemorph between recently collected isolates and older isolates may reflect a general shift in the local mildew population as a result of exposure to this chemical since its introduction in the early 1970s.

The distributions of sensitivity reactions to fenpropimorph and fenpropidin, with a significant proportion of highly sensitive isolates, may reflect the natural variation within the population or a slight shift towards reduced sensitivity. Fenpropimorph has been widely used in south-east Scotland since the early 1980s, but fenpropidin has been used only since 1986.

The history of the powdery mildew isolates incorporated in the screening programme was recorded on receipt, detailing source and any previous chemical association. There was no apparent correlation between history of fungicide contact and sensitivity to the tested fungicides. Lorenz and Pommer (1984), working with wheat powdery mildew, also found sensitivity to fenpropimorph to be independent of previous fungicide treatment.

In some tests low concentrations of fungicide were found to stimulate the growth and sporulation of the isolates. This peculiarity was reproducible and was not an artefact of the experiment. Such stimulation has been observed previously in sensitivity tests on barley powdery mildew (Williamson, 1983) and other fungi (eg Boyle *et al*, 1988).

ACKNOWLEDGEMENTS

We are grateful to Mr E. A. Hunter of the Scottish Agricultural Statistics Service for helpful advice. This work was funded by the Home-Grown Cereals Authority.

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SELECTION DYNAMICS IN TRIAZOLE TREATED POPULATIONS OF ERYSIPHE GRAMINIS ON BARLEY

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ABSTRACT

The changes in the proportion of sensitive (s) and triazole-resistant (r) subpopulations of E. graminis f.sp. hordei were evaluated in discontinuous epidemics over five generations each treated with triazole fungicides. Barley cv. Golden Promise was inoculated with s:r conidium mixtures of initially 1:1, 2:1 or 9:1 and incubated under growth chamber conditions for one week. In untreated populations the r proportion increased only to a limited extent never exceeding more than 50 %, whereas in triazole treated populations the r proportion increased to 100% after 2 to 5 generations depending on the initial r proportion and the fungicide concentration. After discontinuation of fungicide selection, the proportion of the r subpopulation slightly decreased. The selection process was slowed down by both lower fungicide concentrations and decreased r proportions in the initial generation. In "multistrain" mixtures (s:s':r=1:1:1) the r subpopulation did not dominate even after 5 generations each treated with triazoles.

INTRODUCTION

Triazole fungicides have been used intensively for many years to control diseases of cereals. In most countries disease control is still excellent, except in some areas of severe powdery mildew attack (Germany, England) which may be controlled only to a limited degree. As early as 1984, Wolfe et al. reported on reduced sensitivity of Erysiphe graminis populations of barley to triazoles in England. Since then several researchers documented a gradual shift towards lower triazole sensitivity of powdery mildew populations all over Europe and unsatisfactory levels of disease control by triazoles were attributed to increased proportions of resistant subpopulations. In contrast to some other fungicide classes, triazole resistance is of polygenic nature and, therefore, selection dynamics may be different from that of phenylamides or bezimidazoles. The aim of this investigation is to estimate the speed of resistance build-up in mixed populations of E. graminis f.sp. hordei treated over several generations with different concentrations of various triazole fungicides.

MATERIAL AND METHODS

Three different isolates of E. graminis f.sp. hordei were used with a different sensitivity to triazole fungicides (isolates s, s', r, Table 1). In order to single out the strains in a mixture, the difference in virulence was used, tested on barley varieties with known resistance genes. Golden Promise (GP) was sensitive to all three isolates, whereas Koral (K), Mammut (M), and Lerche (L) were sensitive to only one of the isolates, i.e. to the s, s', and r isolate, respectively (Table 1). The virulence analysis was done according to Wolfe and Schwarzbach (1975) on detached barley leaves inoculated in a settling tower with dry spores and incubated on water agar for 7 days at 18° C. The same method as used for the virulence analysis was also used for evaluating the changes in the proportion of sensitive and triazole resistant subpopulations in discontinuous epidemics of E. graminis. One week old barley plants cv. Golden Promise were sprayed with fun-

gicide suspensions, then inoculated with a s:r conidium mixture of initally 1:1, 2:1 or 9:1 (generation 0) and incubated under growth chamber conditions for 7 days. The spores of generation 1 were used to inoculate both a new set of treated barley plants cv. Golden Promise and detached leaves of all four barley varieties. On the treated plants the next fungus generation was produced, whereas on the detached leaves the proportions of the s, s′ and r subpopulations were analysed. This procedure was repeated over five generations. Disease severity was estimated visually as percent leaf area covered with pustules independently on each variety. Percent r and s in a "two strain" population was calculated according to the following formulas:

$\% r_o = 100\ L/GP$ $\quad \% s_o = 100\ K/GP$ $\quad \% r = [\% r_o + (100 - \% s_o)]/2$

L,K and GP are disease severities (%) on Lerche, Koral, and Golden Promise, respectively; $\% r_o$ and $\% s_o$ are the proportions of the populations calculated on the basis of disease severity, whereas % r is the mean proportion calculated on the basis of r_o and s_o (mean variation of 10%). For a "three strain" population the corresponding formulas are:

$\% r_o = 100\ L/GP$ $\quad \% s_o = 100K/GP$ $\quad \%s'_o = 100\ M/GP$
$\% r = (\% r_o + \% r_x)/2$

The term r_x is an unknown amount, calculated as follows:

$\% r_x = \% r_o - \% s'_o + \% s'_x$

TABLE 1. Characterisation of E. graminis f.sp. hordei isolates

	isolate s	isolate s′	isolate r
sensitivity to fungicide (EC 10/50/90; mg AI/l)			
cyproconazole	0.1/0.7/3.5	0.2/0.9/3.5	0.4/4.4/55
propiconazole	<0.01/0.1/0.9	nt /nt /nt	0.1/1.3/11
triadimenol	0.1/0.5/2.5	nt /nt /nt	0.3/4.5/64
fenpropimorph	0.01/0.1/1.3	nt /nt /nt	0.04/0.3/2.8
virulence genes (V), analysed on 10 barley varieties			
present	g,h,k,**v**,a9,**a13**	g,h,k,v,**a6**,a7,a12	g,h,**k**,v,**a7**,a9,a12
symptoms on barley variety (resistance genes Ml)			
present	GP,K	GP,M	GP,L
fitness parameter			
spore production [a]	2389	2693	2830
colony size [b]	9.2	8.5	10.5
disease efficiency [c]	30	20	23
latent period [d]	87	75	80

GP = Golden Promise (no resistance genes); K = Koral (v, a13); L = Lerche (k,a7); M = Mammut (a6); a) spores/colony; b) mm^2 (a and b: 13 d after inoculation); c) number of colonies per 100 spores applied; d) percent sporulating colonies 119 h after inoculation; nt = not tested

RESULTS AND DISCUSSION

Epidemics without fungicide selection or treated with fenpropimorph

In "two strain" populations (s:r = 1:1) not exposed to any fungicide selection (Figure 1) the proportion of the two isolates of originally 50 % each remained fairly stable over five generations (variation between 43 % and 60 %) indicating similar fitness properties of the two isolates. In s:r = 9:1 mixtures the r proportion slightly increased from orginally 10 % to at most 39 % in the fifth generation (data not shown) suggesting a slightly higher fitness of the r isolate. In "three strain" populations (s:s':r = 1:1:1), the r proportion of initially 33 % varied in the following generations between 33 % and 60 %, whereas the s proportion slightly decreased to a range between 12 % and 28 % (Figure 5A). Specific investigations confirmed the slightly lower fitness of the s isolate which was expressed in a somewhat smaller spore production and colony size (Table 1). In "two strain" populations (s:r = 1:1) treated with fenpropimorph no significant change in the proportion of s and r occured over five generations (Figure 1) indicating that no selection was exerted by this morpholine.

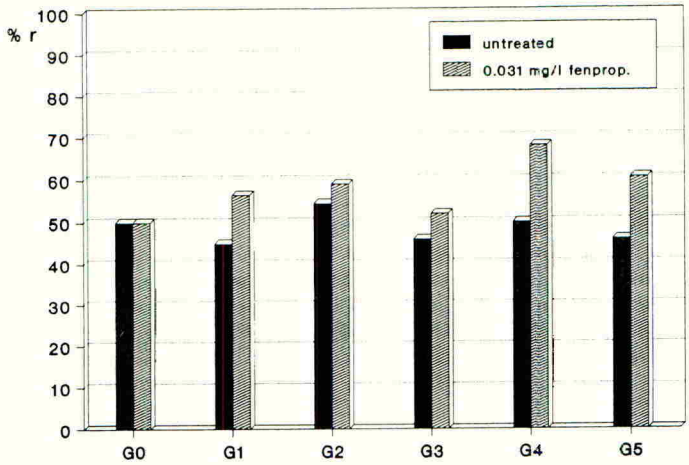
Epidemics treated with different concentrations of triazoles

As expected, all triazoles tested imposed a selection on the population causing an increase of the r subpopulation to various degrees in the following generations (Figures 2-5, Table 2). In "two strain" populations (s:r = 1:1) treated with 2 and 0.5 mg/l of cyproconazole, the r subpopulation dominated (>90%) after three generations, whereas at 0.125 mg/l the selection process was delayed for one generation more (Figure 2). Thus, the selection process was clearly slowed down by lowering the fungicide concentration supporting many models predicting this behaviour for different types of fungicides. The same obervation was made using a s:r = 9:1 mixture (Figure 3) in which the whole selection process progressed more slowly because of the lower initial amount of the r subpopulation. At 2.0 and 0.5 mg/l of cyproconazole, the r population reached 90 % after four generations, whereas at 0.125 and 0.031 mg/l the same level was reached after five generations. In the first four generations the progress of the selection process was clearly slowed down by lower fungicide concentrations.

After a selection period of five generations, each exposed to 0.5 mg/l of cyproconazole or triadimenol, there was still a small proportion (0.5 - 5 %) of the s subpopulation left which, after the entire population was transferred to untreated plants for another four generations, gradually recovered and increased to 10 - 23 % in the ninth generation (Figure 4). This behaviour suggests that almost completely triazole resistant populations do not necessarily remain resistant after fungicide use has been terminated, in contrast to other classes of fungicides (e.g. benzimidazoles). The rate of 0.5 mg/l of cyproconazole corresponds to about an EC 50 for the s isolate and to an EC 10 for the r strain (Table 1). Despite this rather unfavourable selection pressure for the s subpopulation it recovered to a certain degree after the fungicide treatment had been stopped. Thus, sublethal concentrations certainly do not favour resistant subpopulations. As already documented in Figure 1, the s isolate cannot be significantly less fit than the r strain, otherwise it would not have started to recover (Figure 4).

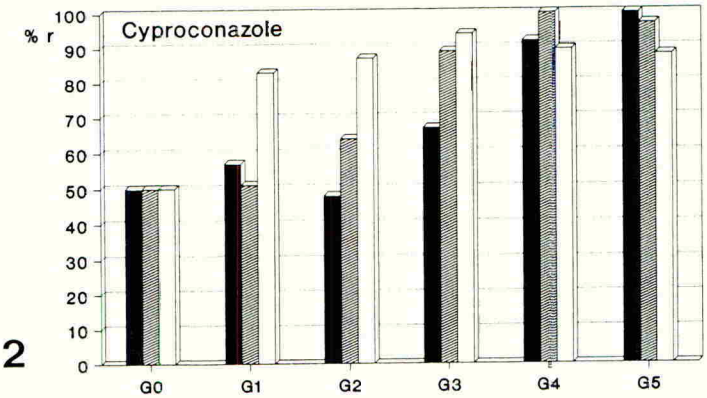
Closer to real field populations than "two strain" are "three strain" mixtures. In a s:s':r= 1:1:1 mixture treated with 0.5 mg/l of cyproconazole the r subpopulation increased from initially 33 % to about 65 % in the third and fourth generations but decreased again in the next two genera-

FIGURE 1. Proportion of r (%) in a s:r=1:1 mixture with and without fungicide treatment (fenprop. = fenpropimorph) over 5 generations (G1-G5).



FIGURES 2 and 3. Proportion of r (%) in a s:r=1:1 mixutre (Fig. 2) or a s:r=9:1 mixture (Fig. 3) treated with cyproconazole over five generations (G1-G5).

2



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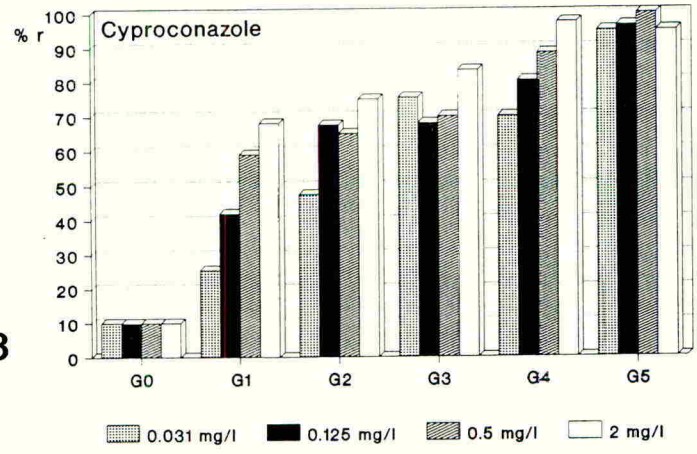


FIGURE 4. Proportion of r (%) in a s:r=1:1 mixture treated with triazoles over 5 generations (G1-G5) and after treatment has been stoped (G6-G9).

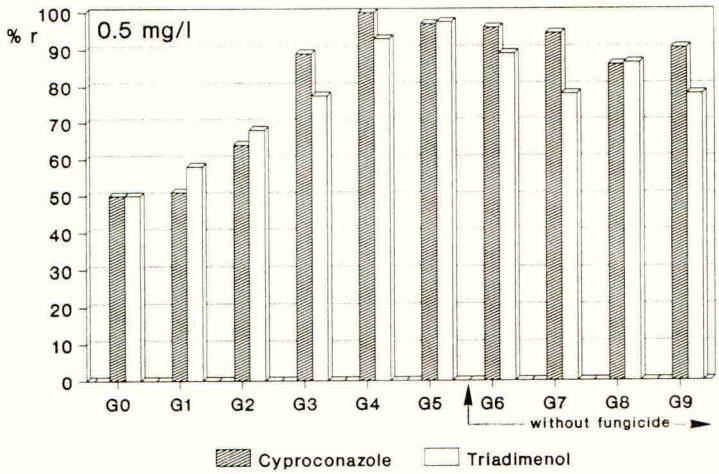
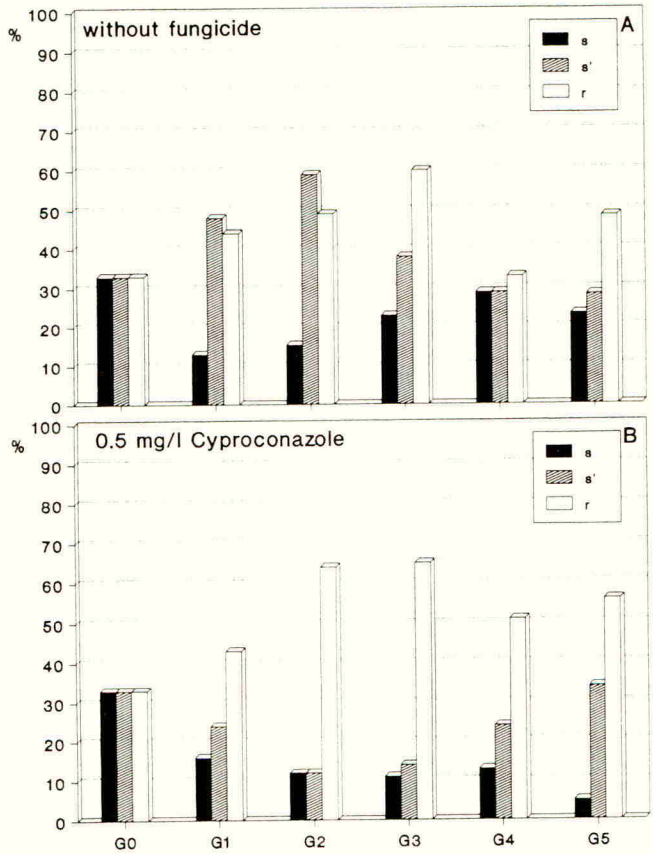


FIGURE 5. Proportion of r (%) in a s:s':r=1:1:1 mixture with and without fungicide treatment over five generations (G1-G5). In G1-G5 the total of s+s'+r is for methodological reasons not always exactly 100.



tions (Figure 5B). Both the s and s' subpopulations decreased to a certain degree but were stabilised on a level of about 10 % and 25 %, respectively. In the fungicide treated population (Figure 5B) the r subpopulation did not increase more than in the untreated population (Figure 5A) but the ratio between the strains changed with time. Obviously, there is a strong internal competition between the three strains preventing a build-up of any dominant subpopulation selected out by fungicides. Thus, in field populations the fungicide selection process exerted by triazoles is expected to be less pronounced than in discontinuous epidemics under greenhouse conditions.

The three triazoles cyproconazole, propiconazole and triadimenol, considered to be of the third , second and first "triazole generation", respectively, were compared for their potential to select triazole resistant subpopulations (Table 2). There were some variations in proportion of the r subpopulations in certain generations selected by the different triazoles (e.g. G_1: 45 % for propiconazole and 57 % for triadimenol; G_2: 50 % for propiconazole and 68 % for triadimenol; G_3: 77 % for triadimenol and 85 % for propiconazole) but the overall selection process was about the same for all three triazoles.

TABLE 2. Proportion of r (%) in a s:r=2:1 mixture treated with 0.5 mg/l of different triazoles over five generations (G_1 - G_5)

	proportion of r (%)					
compound	G_o	G_1	G_2	G_3	G_4	G_5
cyproconazole	33	48	66	80	96	97
propiconazole	33	45	50	85	93	95
triadimenol	33	57	68	77	92	96

CONCLUSIONS

The three isolates (s, s', r) used in this study did not differ significantly in their fitness. They exerted strong internal competition and none of them dominated the untreated population even after five generations. On the other hand, triazole treatments of the population caused a build-up of the r subpopulation. The selection process was slowed down by both lower fungicide concentrations and decreased initial r proportions; in "multistrain" mixtures the r subpopulation did not dominate even not after five generations. Anti-resistance strategies are needed to prevent resistance build-up by triazoles to unacceptable levels. These strategies may include non cross-resistant fungicides like morpholines.

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FLUTRIAFOL, ETHIRIMOL AND THIABENDAZOLE SEED TREATMENT - AN UPDATE ON FIELD PERFORMANCE AND RESISTANCE MONITORING

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ABSTRACT

Seed treatment with a mixture of flutriafol, ethirimol and thiabendazole continues to give a high level of control of barley powdery mildew in both winter and spring cultivars. The product also gives beneficial growth effects, especially in winter barley. Continued monitoring studies have shown that the sensitivity of mildew populations to both ethirimol and the DMI group has not changed significantly since its introduction. Recent trials demonstrated a high level of control of mildew across a wide range of both spring and winter barley cultivars. In spring barley, mildew control led to significant increases in yield.

INTRODUCTION

As UK farmers strive to produce maximum returns from both winter and spring barley, their use of high rates of fertiliser, coupled with early sowing, has resulted in crops being subject to heavy disease pressure. One of the most important of these diseases in the UK is powdery mildew caused by Erysiphe graminis.

In 1984, 'Ferrax' (flutriafol + ethirimol + thiabendazole) was introduced as a seed treatment for the control of the major seed-borne, soil-borne and early foliar diseases of barley (Northwood et al, 1984). By combining the activity of a DMI (an inhibitor of fungal ergosterol biosynthesis at the demethylation step) of the triazole group, flutriafol, with a hydroxypyrimidine, ethirimol, a dual mode of action against powdery mildew was achieved. Thiabendazole was added to improve the control of Fusarium spp.

Six years of commercial experience have demonstrated its effectiveness and shown there are additional, useful, early growth benefits associated with its use. Monitoring work has been undertaken to study the sensitivity of powdery mildew populations to both triazoles and ethirimol so that any adverse changes noted may be used to influence technical management of the product.

This paper describes recent work by ICI Agrochemicals where mildew control, growth benefits and sensitivity of mildew populations to the product were studied across a large number of commercially available cultivars in England and Scotland.

METHODS AND MATERIALS

Two different types of field trial were undertaken to test the field performance of the flutriafol + ethirimol + thiabendazole formulation compared to a mercury standard.

Spring Barley

Trials were fully replicated using a randomised block design with an individual plot size of 72 m^2. Mildew assessments were made according to recognised guidelines on 10 leaves per plot.

Winter Barley

Trials were of an unreplicated split field design. They were laid down away from field headlands and used a plot size of between 0.5 and 1.0 hectare. Assessments of disease levels were made taking 25 leaves per plot. Measurements of crop morphology were sometimes carried out. 3 x 1 m rows of crop per plot were sampled at random and assessments made of the number of plants per metre row, the number of tillers per plants and individual tiller and whole plant weights.

The sensitivities of mildew populations to triazole fungicides and ethirimol were monitored using methods described by Heaney et al, 1986.

A simple statistical comparison of means test was used to compare yields. Values followed by a common letter are not significantly different (P = 0.05).

RESULTS AND DISCUSSION

Triazole-based systemic seed treatments produce plant growth benefits in winter barley by increasing both plant and tiller weight (Table 1). Previous trials had shown this, but also indicated that an additional effect was a reduction in the number of tillers per plant (Noon et al, 1988). Work undertaken in early 1990 showed that rather than giving a significant decrease in tillers per plant, the flutriafol formulation gave a small increase. This apparent contradiction was attributable to differences in the weather conditions over the two seasons when observations were made.

The winter of 1986/87 was relatively severe with frequent frosts. As a result, the mercury treated plots were subject to plant loss. By comparison, plants treated with the flutriafol formulation were more vigorous and were better able to withstand the cold weather. When the assessments were made, the plants in the plots treated with mercury, being of lower density, were compensating by producing a greater number of tillers. The overall effect was an apparent reduction in the number of tillers per plant with the systemic seed treatment.

During the winter of 1989/90 the weather was extremely mild. This encouraged the development of mildew on the mercury treated plots. As a result, the crop was under stress. Rather than causing actual plant loss, however, the disease reduced the tillering capacity of the plants. By comparison, the plots treated with the flutriafol formulation were not subject to tiller loss.

TABLE 1. Plant growth benefits in winter barley, spring 1990* (expressed as % of mercury treatment).

Treatment	Rate (mg ai/ kg seed)	Plants /m row	Tiller number /plant	Plant weight	Tiller weight	Weight of plants /m row
Flutriafol + ethirimol + thiabendazole	150 2000 50	103	111	124	117	126
Mercury (actual figures)	22	100 (54.7)	100 (3.6)	100 (2.2g)	100 (0.7g)	100 (112.5g)

Assessments were made 19-24 weeks after drilling.

* Mean data from 26 trials across the following cultivars:

Halcyon, Magie, Marinka, Maris Otter, Melusine, Panda, Pastoral, Pipkin, Plaisant, Puffin, Torrent.

Good control of mildew was achieved 7 weeks after drilling even though in some trials the disease pressure was exceptionally high (Table 2). By 10 weeks after drilling there was only a slight drop in the level of mildew control. Not all trials were assessed 10 weeks after drilling since in some, particularly the later drilled ones, mildew on both treatments was reduced due to the onset of colder weather.

TABLE 2. % Mildew control in winter barley, 1989/90.

Cultivar	7 weeks after drilling % control*	No. trials	10 weeks after drilling % control*	No. trials
Frolic	91 (44)	3	-	-
Halcyon	88 (15)	2	-	-
Kira	96 (30)	2	-	-
Magie	85 (20)	7	74 (16)	4
Marinka	82 (10)	6	76 (20)	3
Maris Otter	86 (23)	4	91 (14)	2
Panda	100 (1)	1	-	-
Pastoral	89 (35)	3	70 (10)	1
Plaisant	90 (14)	3	77 (27)	1
Pipkin	76 (10)	4	68 (49)	3
Puffin	67 (5)	1	57 (4)	1
Torrent	93 (6)	4	89 (15)	2

* Disease levels on mercury treated plots are in parentheses.

Later drilled trials had lower levels of mildew on the mercury treated plots when assessed 7 weeks after drilling (Table 3). The mildew control given by the flutriafol formulation was similar regardless of date of drilling.

TABLE 3. Effect of date of drilling on mildew control+, 1989/90.

Month of drilling	September	October
Mean date of drilling	22-9-89	10-10-89
% control of mildew 7 weeks after drilling*	88 (25)	86 (10)

* Disease levels on mercury treated plots are in parentheses.

+ Means of 15 trials for each month, made up of an equal number of trials on each cultivar.

In the early 1980's the use of single ingredient products for the control of barley powdery mildew was questioned. This was due to changes in the sensitivity of the mildew population to fungicides. Initially, a build-up of resistance to ethirimol occurred in the 1970's (Shephard et al, 1975). Later, resistance to the DMI group developed (Butters et al, 1984). To counteract this, the use of product alternation and the mixing of fungicides with different modes of action were introduced. This led to a stabilisation of the mildew population (Heaney et al, 1988).

Table 4 shows that despite a small drop in sensitivity to ethirimol immediately after the introduction of the hydroxypyrimidine + DMI mixture, mildew sensitivity to ethirimol has not changed significantly since 1986. Sensitivity to ethirimol is now at a level substantially higher than it was in 1974 when the single ingredient product was in use.

TABLE 4. Changes in sensitivity to ethirimol in barley powdery mildew populations in England and Scotland, 1974-1989.

Year	% of population in each sensitivity grade*						Mean sensitivity
	<6 (least sensitive)	6-9	9-12	12-15	15-17	>17 (most sensitive)	
1974	1	16	60	13	7	2	10.8
1984	1	4	10	26	27	32	15.1
1986	0	0	3	54	36	7	14.8
1987	0	1	20	56	21	2	13.5
1988	0	0	8	65	24	3	14.0
1989	0	0	9	70	19	2	13.7

* After Shephard et al, 1975.

Sensitivity values to triadimenol (the test DMI fungicide) have been fairly stable since 1986, suggesting that resistance to DMI fungicides is not increasing further (Table 5).

TABLE 5. Changes in sensitivity to triadimenol in barley powdery mildew populations in England and Scotland, 1986-1989.

Year	% of population in each sensitivity grade					Mean sensitivity
	<5 (least sensitive)	5-9	9-13	13-17	>17 (most sensitive)	
1986	19	40	37	4	0	8.6
1987	31	38	26	5	1	7.0
1988	9	49	40	1	0	8.1
1989	23	56	12	4.5	4.5	7.5

Recent trials on spring barley in Scotland have confirmed the mildew control and yield benefits associated with use of the systemic mixture (Tables 6 and 7). They also show that a follow-up foliar mildewicide may be unnecessary unless the crop is subject to season-long moderate to heavy disease pressure.

TABLE 6. % Control of powdery mildew on four cultivars of spring barley in Scotland, 1989.

Weeks after drilling	Trial location and % control of powdery mildew*			
	Midlothian cv. Golf	Midlothian cv. Klaxon	Kinross cv. Triumph	Kinross cv. Golden Promise
5-7	100 (2.0)	97 (0.9)	37 (3.3)	82 (4.6)
7-8	77 (15.9)	68 (6.6)	71 (2.6)	60 (5.7)
8-9	79 (8.0)	72 (1.5)	50 (2.7)	67 (8.2)
9-10	93 (1.3)	93 (0.4)	70 (8.6)	52 (14.9)
11	58 (4.3)	57 (2.3)	76 (4.8)	77 (23.0)
13	72 (16.7)	53 (3.2)	94 (0.9)	77 (9.6)

* Assessments were of leaf 3 or 4. Disease levels on mercury treated plots are in parentheses.

CONCLUSION

A mixture of flutriafol, ethirimol and thiabendazole has now been in widespread use on barley crops in the United Kingdom for six years. It is still the only commercially available fungicidal seed treatment that combines the activity of two mildewicides with different modes of action. During this period, it has maintained a high level of action against barley powdery mildew, supporting the view that mixtures play an important role in maintaining the efficacy of their component active ingredients.

TABLE 7. Yield responses, as % untreated, on four cultivars of spring barley in Scotland, 1989.

Seed treatment	Fungicide treatment Rate (mg ai/ kg seed)	Foliar spray	Rate (g ai /ha)	Trial location and % yield response Mid-lothian cv. Golf	Mid-lothian cv. Klaxon	Kinross cv. Triumph	Kinross cv. Golden Promise
Flutriafol + ethirimol + thiabendazole	150 2000 50			108 c	106	108 a	111 b
Flutriafol + ethirimol + thiabendazole	150 2000 50	Fen-propi-din	750	110 c	103	108 a	123 a
Mercury (actual yield t/ha)	22			100 d (4.47)	100 (5.35)	100 b (4.11)	100 c (3.41)

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BIOCHEMICAL ASSAYS FOR INSECTICIDE RESISTANCE: STRENGTHS AND LIMITATIONS

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ABSTRACT

Possible implications of allelic variation and multifactorial resistance for biochemical monitoring of resistance were investigated by comparing patterns of acetylcholinesterase insensitivity to organophosphorus insecticides in houseflies (Musca domestica), disclosed by a microplate assay, with cross-resistance spectra obtained by bioassays. Results emphasised the value of biochemical tests for analysing these genetic phenomena, but highlighted potential pitfalls when such tests are used indiscriminately.

INTRODUCTION

Biochemical and immunological assays that diagnose qualitative or quantitative differences in enzymes implicated in insecticide resistance offer tremendous scope for improving the speed and sensitivity of resistance monitoring. By distinguishing unambiguously between susceptible and resistant insects, such assays overcome some of the statistical constraints on toxicological tests and reduce the sample sizes needed to detect resistance at a given level of probability (Roush & Miller, 1986). By increasing throughput, they also have the potential to detect resistance at the low gene frequencies necessary for effective implementation of many management tactics (eg. Curtis, 1987).

Whilst recognising these advantages, it is also important to appreciate the limitations of biochemical tests for large-scale monitoring programmes. This approach is clearly only relevant when changes detected biochemically correlate with differences in toxicological response (Devonshire, 1990). Even when a causal relationship has been established, as with the mechanism of acetylcholinesterase (AChE) insensitivity to organophosphorus (OP) and carbamate insecticides (reviewed by Devonshire, 1980), there is a danger that other alleles at this locus or additional mechanisms may obscure this relationship for field populations (Sawicki, 1987; Denholm, 1990). We explore this possibility here by comparing patterns of AChE insensitivity in well-defined strains of houseflies (Musca domestica), disclosed by a kinetic microplate assay, with cross-resistance data obtained from topical application tests.

MATERIALS AND METHODS

Housefly strains

The five strains examined differed in genetic composition at OP-resistance loci (Table 1). All were homozygous for a single AChE variant, showing either base-line sensitivity to OPs (S variant) or contrasting patterns of insensitivity to these compounds (CH2 and 49R variants; Moores

et al., 1988a). In addition, all were homozygous for either the presence (+) or absence (-) of $E_{0.39}$, an esterase implicated in resistance to both OPs and pyrethroids (Sawicki et al., 1984). For reasons given later, strains CH2, 7038 and 7988 were far less likely to possess other unidentified OP-resistance factors than 49R.

TABLE 1. Genetic composition of five housefly strains.

Strain	OP-resistance locus AChE	$E_{0.39}$	Other
Cooper	S	-	-
CH2	CH2	-	-
49R	49R	-	?
7038	S	+	-
7988	CH2	+	-

Insecticides and bioassays

The seven insecticides tested (Table 2) included most OPs and carbamates used widely in Europe for fly control. For topical application tests, 3-4 day old adult females were each treated with 0.5ul of technical grade insecticide in acetone. Each dose was applied to two replicates of 15 flies, and mortality was scored after holding at 20°C for 48h. Resistance in strains CH2, 49R, 7038 and 7988 was expressed by transforming LD_{50} values obtained by probit analysis into resistance factors relative to the corresponding Cooper LD_{50}.

Characterisation of AChE insensitivity

AChE insensitivity to these seven insecticides or their active metabolites was measured using acetylthiocholine as substrate, and detecting the released thiol colorimetrically by its reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). The procedure was a slight modification of that given by Moores et al. (1988a). To enable seven inhibitors to be assayed simultaneously against each individual, 16 insects of the required strain were homogenised in phosphate/Triton (0.1M phosphate buffer, pH 7.5 containing 1% Triton X-100) in separate wells of a microtitre plate (NUNC), and two separate 50ul fractions of homogenate were diluted with a further 200ul phosphate/Triton. Eight 50ul replicate aliquots (each equivalent to 0.05 of a fly) of this dilution were then assayed either without inhibitor or with a diagnostic concentration of each of the seven inhibitors. These concentrations (listed in Table 2) were established from preliminary experiments as the lowest ones giving almost complete inhibition of the S enzyme in the Cooper strain.

Reactions monitored over 10 min by a V_{max} kinetic microplate reader (Molecular Devices) were summarised by fitting linear regressions to the absorbance-time data for each well. To compensate for differences in the catalytic efficiency of the three AChE variants, the slope of each inhibited reaction was expressed as a percentage of that for the same insect in the absence of inhibitor (Moores et al., 1988a). This measure of

'% activity remaining' during inhibition provided a qualitative means of diagnosing insensitivity to each compound relative to the response of the S variant. Further work to obtain quantitative measures of insensitivity for a more precise comparison with bioassay data is in progress.

RESULTS AND DISCUSSION

AChE insensitivity profiles of Strains CH2 and 49R

Resistance caused by mutant AChE does not involve just one insensitive enzyme. Several forms with contrasting patterns of insensitivity occur (Devonshire & Moores, 1984), often within single species and sometimes within single populations (Moores et al., 1988b and unpublished data). Initial evidence for distinguishing the CH2 and 49R variants came from work with just two inhibitors; whereas the CH2 enzyme showed insensitivity to dichlorvos but not to azamethiphos, 49R showed the converse pattern (Moores et al., 1988a). Consequently, a bivariate plot of adjusted inhibition rates with these compounds clearly resolved all six genotypic combinations of the S, CH2 and 49R alleles. The present study encompassing more inhibitors supported this finding for AChE homozygotes (Figure 1a), but disclosed other clearcut differences between the enzymes. In particular, paraoxon and omethoate gave a similar reciprocal pattern of insensitivity and achieved an equally effective degree of separation (Figure 1b).

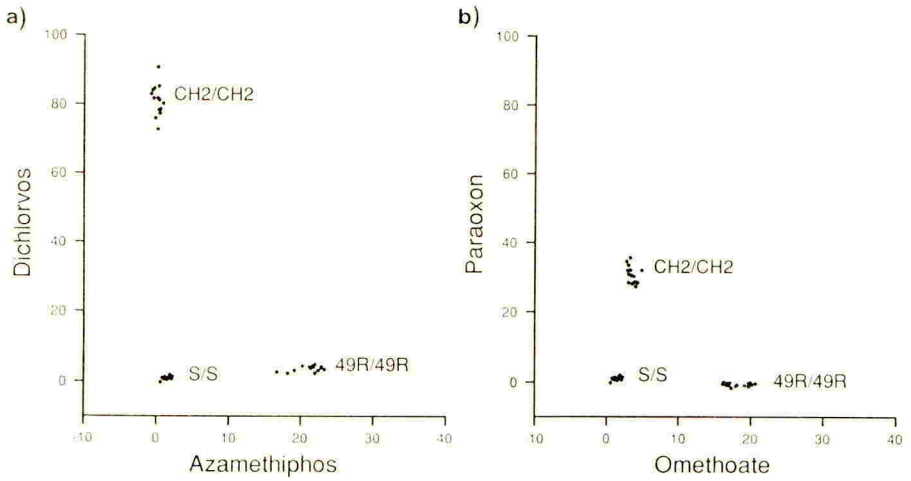

Figure 1. Bivariate plots of mean % activity remaining during inhibition of S, CH2 and 49R homozygotes with (a) 10uM dichlorvos and 0.2uM azamethiphos, and (b) 40uM paraoxon and 1mM omethoate. 48 individuals are represented on each plot.

Full profiles for CH2 and 49R showing the presence (+) or absence (-) of detectable insensitivity to each inhibitor are listed in Table 2. Only two of the seven compounds (malaoxon and fenitroxon) correctly diagnosed both insensitive enzymes; reliance on any one of the remainder would either underrate the importance of this mechanism in populations containing both

alleles or, in the case of methomyl, fail to disclose it altogether. Hence although the microplate assay is ideally suited for resolving allelic variation and monitoring well-characterised enzymes, it should not be used indiscriminately to document AChE insensitivity in field populations of unknown allelic composition.

Relationship between AChE insensitivity and cross-resistance patterns

Far from possessing just one major factor of resistance, insects subjected to intensive chemical control often accumulate an array of mechanisms conferring resistance to the same toxins (eg. Sawicki, 1975; Raymond et al., 1986). In houseflies, for example, AChE insensitivity is only one component of an intricate multifactorial system of resistance to OPs that also includes reduced cuticular penetration and several detoxification processes (reviewed by Oppenoorth, 1985). This may considerably undermine the ability of one mechanism-specific assay to describe changes in the phenotypic tolerance of field populations. Cross-resistance data for strains CH2, 49R and 7988 exemplify the problem.

Strain CH2

The CH2 variant was originally isolated in Holland by chromosome substitution and extensive backcrossing, supplemented by biochemical analyses aimed specifically at eliminating other OP-resistance factors (Oppenoorth et al., 1977). At Rothamsted, the strain containing this enzyme was backcrossed for a further four generations to Cooper to maximise the prospect of isolating the allele in a fully susceptible genetic background. As a result, its AChE insensitivity profile accorded exactly with the range of insecticide precursors resisted more than 2-fold in bioassays (Table 2). The microplate assay therefore gave an accurate description of the toxicological response of this highly-inbred strain.

TABLE 2. Comparison of AChE insensitivity profiles and cross-resistance in strains CH2 and 49R.

AChE insensitivity				Resistance factor		
Inhibitor	Conc[1]	CH2	49R	Insecticide	CH2	49R
Dichlorvos	10	+	-	Trichlorphon	5.4	8.7
Paraoxon	40	+	-	Parathion	5.0	3.9
Malaoxon	10	+	+	Malathion	2.5	10
Omethoate	1000	-	+	Dimethoate	1.2	13
Fenitroxon	100	+	+	Fenitrothion	5.3	4.4
Azamethiphos	0.2	-	+	Azamethiphos	1.1	3.3
Methomyl	100	-	-	Methomyl	1.2	2.9

[1]Concentration of inhibitor (uM) used to diagnose insensitivity

Strain 49R

In comparison, 49R had a much less intricate selection history, being derived from a multiresistant Danish field strain ($49r_2b$) simply as a single-pair progeny consistently showing reduced sensitivity to azamethiphos. Apart from two generations of backcrossing to Cooper, there was no deliberate attempt to exclude additional mechanisms undoubtedly

present in the base population. The broad-spectrum resistance of 49R to OPs and carbamates, which showed no apparent correlation with its insensitivity profile (Table 2), can therefore be attributed to interactions between AChE and other, as yet unidentified OP-resistance loci.

Strain 7988

The importance of interactions between loci was studied more directly by constructing a strain homozygous for two well-characterised mechanisms in a susceptible genetic background. 7988 possessed both the CH2 allele for AChE insensitivity and the $E_{0.39}$ esterase gene transferred from strain 7038. $E_{0.39}$ alone conferred only moderate (6 to 12-fold) resistance to trichlorphon and malathion, but interacted with CH2 to give very strong resistance to these compounds (Table 3). Hence there were very marked differences in the magnitude and ranking of resistance factors between 7988 and CH2 having identical patterns of AChE insensitivity.

TABLE 3. Resistance factors for strains CH2, 7038 and 7988.

Insecticide	Strain CH2	7038	7988
Trichlorphon	5.4	12	92
Parathion	5.0	1.7	7.2
Malathion	2.5	5.8	53
Dimethoate	1.2	1.5	2.5
Fenitrothion	5.3	2.6	10
Azamethiphos	1.1	1.3	2.7

The spread of multifactorial resistance is probably the most serious constraint on biochemical monitoring methods. With sustained selection pressure, assays specific to single mechanisms may become increasingly unreliable as measures of phenotypic tolerance, and as indicators of the likely field performance of pesticides. Even when there is strong evidence for a single, widespread mechanism of resistance, as in UK populations of the aphid Myzus persicae (Devonshire, 1990), constant scrutiny is required to ensure that an assay remains relevant over time and applicable to other geographical areas.

CONCLUSIONS

Conflicts between the specificity and practical utility of biochemical assays can only be resolved by recognising their strengths and limitations for resistance monitoring. As research tools, these techniques offer unparalleled opportunities for resolving allelic variation, studying interactions between resistance loci, and analysing the build-up of resistance under controlled conditions. But their uncritical use against field populations is clearly fraught with hazard. Used with discretion, biochemical assays can complement toxicological tests but will never wholly supplant them.

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THE IMPACT OF PHOSPHINE RESISTANCE ON THE CONTROL OF INSECTS IN STORED GRAIN BY PHOSPHINE FUMIGATION

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ABSTRACT

Laboratory studies on the toxicity of phosphine (hydrogen phosphide) on adults and immature stages of susceptible and phosphine resistant strains of four species of beetles indicate large differences in the dosage required for control. Two bulks of floor-stored wheat were fumigated, one dosed conventionally and the other using an experimental method. The latter technique gave better fumigant distribution and retention.

A comparison of the doses obtained in the fumigations with those necessary to control the various strains of insects suggests that the use of the conventional dosing method will allow survival of immature stages of susceptible strains. The improved dosing method will control all susceptible strains except immature *Sitophilus* spp. but is inadequate to control resistant strains except that of *Oryzaephilus surinamensis*.

In order to guarantee the control of resistant insects and to avoid further selection pressure for resistance, radical changes in the method of applying phosphine to grain are advocated.

INTRODUCTION

Grain is stored in the UK in a diversity of structures, including galvanised steel bins, concrete silos and on the floor in purpose built stores, converted aircraft hangers or in other buildings. Most but not all feature a ventilation system. This diversity coupled with the prevailing cool, damp weather conditions, creates difficulty in devising a general set of recommendations for the use of phosphine as a fumigant. From a biological viewpoint further complexities arise because of the very wide differences in tolerance to phosphine between stages of the same species and between different strains and species. There are also some unique toxicological properties when phosphine is applied to insects, temperature having a profound effect. The dosage required increases dramatically as the temperature of treatment lowers until the temperature itself is low enough to be lethal.

The impact of resistance on the dosage required can be very great in adult insects (Mills, 1983) and in other developmental stages (Price and Mills, 1988). The exposure time is more important than the concentration for both susceptible and resistant adults (Winks, 1984; Winks and Waterford, 1986). In *Rhyzopertha dominica*, resistant adults exhibit an active exclusion mechanism (Price, 1984) which delays uptake.

For actively developing stages of many species the minimum exposure

period required for control is governed by the developmental rate of eggs or pupae. Phases of high natural tolerance can be bridged by longer exposures as development continues in the presence of phosphine both for susceptible strains (Hole, *et al.*, 1976) and for resistant strains (Price and Mills, 1988).

The units for phosphine dosage are best expressed as concentration-time products (cts) over a defined exposure period. The ct is the area under a curve of concentration (g/m^3) plotted against exposure time (h) and this concept of dose holds, provided that the concentration remains above a minimum level for the whole exposure period. Units for cts are gram hours/m^3 (gh/m^3).

Grain in the UK is dried and then cooled down to between 4 and 10°C by aeration. Nevertheless localised infestations often occur in large bulks and require treatment. Phosphine fumigation is the only control method that is currently available for treating a whole bulk *in situ*. Phosphine gas is produced in grain by the action of atmospheric moisture on a formulation of aluminium phosphide powder in sachets or compacted tablets or pellets. The rate of release is governed by temperature and grain moisture content. Gas diffuses through the bulk and is lost mainly by leakage but also by sorption on the grain. The result of these factors is a wide range of cts at different locations and usually with peak concentrations early, and low concentrations below the critical level at the end of long exposures, especially at the periphery of bulks. Under leaky conditions extended generation times would be required to provide an effective exposure and an even distribution of gas through the bulk.

Against this background the control even of susceptible strains can be uncertain. The presence of insects resistant to phosphine could make the situation worse with a higher frequency of control failure, expense and the possibility for further selection for resistance. Taylor (1989) has warned of the threat to phosphine fumigation but hitherto little attempt has been made to obtain adequate data on gas concentration levels from commercial treatments for comparison with the dosages necessary for the control of resistant strains experienced in laboratory studies.

The objectives of the present work are:-

1. To compare doses of phosphine obtained by current fumigation practice and by an improved dosing technique with those required for the control of susceptible and resistant strains and comment on their efficacy.

2. In the light of the comparison to suggest further improvements in fumigation technique to avoid survival of insects and selection pressure for further resistance.

MATERIAL AND METHODS

The insect species studied were the Saw-toothed grain beetle, *Oryzaephilus surinamensis* (O.s.), the Rice weevil, *Sitophilus oryzae* (S.o.), the Rust-red grain beetle, *Cryptolestes ferrugineus* (C.f.) and the Lesser grain borer, *Rhyzopertha dominica* (R.d.). The first three species are common pests in the UK though R.d. is rare.

Toxicity tests on adult insects

These closely followed the FAO Test Method 16 for phosphine resistance (Anon, 1975). Briefly, replicated batches of at least fifty adults aged 3-5 weeks were acclimatised at 15°C and 60% r.h.. Two replicates were fumigated in adapted glass desiccators of known volume which were dosed with phosphine using a gas-tight syringe to give a range of concentrations with appropriate untreated controls. Twenty-hour exposures were run for susceptible strains while for resistant strains exposures lasted for up to 7 days.

Phosphine concentrations were sampled and measured by gas chromatography according to the method of Mills (1983). After treatment adults were held at 25°C and 60% r.h. for 14 days prior to mortality assessment. The mortalities were corrected for control deaths and the dose response data analysed by probit analysis (Finney, 1971) for the different exposure periods.

Toxicity tests on immature insects

Cultures containing large numbers and all developmental stages of the same susceptible and resistant strains were exposed in a 1700 litre fumigation chamber to various phosphine concentrations and exposure periods up to 14 days at 15°C and 60% r.h.. The method of dosing, sampling gas and concentration measurement by gas chromatographic analysis is described by Price and Mills (1988). After treatment, cultures were held at 25°C and 60% r.h. and examined weekly for emerging adults. The number emerging was expressed as a percentage of those in replicate untreated controls and cts for control and various exposures were determined.

Field trials

Two field trials were carried out on small bulks of wheat in flat stores, the first using a conventional dosing method and the other using a new method.

Trial A

A 270 tonne bulk held in the corner of a flat store was used. It measured 7.5m by 16.0m contained by metal walls on three sides and a wooden wall at the front. The grain was 2.7m to 3.0m deep sloping down to 2.0m at the front. It was dosed with a total of 90 sachets (Trademark : Detia GmbH, Laudenbach, FRG), each liberating 11g of phosphine. These yielded 990g or 3.7g/tonne and were distributed in five rows at up to 1.0m depth. The grain was covered with a PVC sheet 500 microns thick, which extended over the wooden wall to the floor. Gas samples were taken at intervals via nylon capillaries inserted at 9 representative locations and depths using partially evacuated glass flasks containing acidified potassium permanganate. These were analysed chemically by the method of Bruce *et al.*, (1962). Grain temperatures ranged from 5 to 12°C with a mean of 10.1°C. The spent sachets were recovered at the end of the treatment which lasted 13 days.

Trial B

A 700-tonne bulk held on a ventilated floor was treated using an experimental method involving the dosing of the ventilation ducts and the grain surface. The wheat was held at the end of a purpose-built store in an

area 50m by 18m. It was contained by 3m metal walls on three sides and a 3.6m concrete wall at the front. The grain was 5m deep at the centre sloping to 1.5m at the corners. The surface was dosed with sachets on a radial pattern from the centre. Eight spokes at equal angles formed a template for the insertion of 26 sachets per spoke to 0.3m depth giving 208 sachets which in total liberated 2288g of phosphine. Three ventilation ducts 6m apart were each dosed with 50 sachets using a plastic probe 18m long. These liberated a total of 1650g of phosphine. The total dose was 3938g or 5.6g/tonne. The dose applied to the ducts was 43% of the total. The ducts were well sealed with a PVC-nylon-PVC trilaminate and masking tape and the grain surface and front wall were covered using three overlapping polyethylene sheets 220 micron thick weighted down at the edges with chain. The gas concentrations were sampled at frequent intervals via nylon capillaries at 22 representative locations and depths which were attached to an automated gas chromatograph in a mobile laboratory. Grain temperatures ranged from 2° to 23°C with a mean of 6.5°C. The fumigation lasted 16 days after which the spent sachets were recovered.

The cts for all positions for both trials were computed by integrating the areas under concentration-time curves for the total exposure periods and for 3 and 8-day periods.

RESULTS

The toxicity tests

Table 1 gives the cts for various exposures required to control susceptible and resistant adults. These are obtained by multiplying the $LD_{99.9}$'s from toxicity tests by the appropriate exposure time in hours.

TABLE 1. Toxicity of PH_3 to adult insects at 15°C 60% r.h.

Species	Exposure hours	Slope ± SE	$LD_{99.9}$ g/m^3	ct gh/m^3
O.s S	20	4.6 ± 0.3	0.152	3.04
S.o S	20	5.0 ± 0.4	0.065	1.30
R.d S	20	5.4 ± 0.6	0.083	1.66
C.f S	20	4.7 ± 0.7	0.155	3.10
O.s R	168	5.2 ± 0.8	0.750	126.0
S.o R	72	3.9 ± 0.2	0.694	50.0
S.o R	168	3.7 ± 1.2	0.150	25.2
R.d R	168	10.8 ± 1.0	0.996	167.0
C.f R	168	6.3 ± 0.5	2.982	501.0

S = susceptible R = resistant

Table 2 gives the cts for various exposures for the control of all immature stages of susceptible and resistant strains as judged from the data of Price and Mills (1988).

TABLE 2. Cts to control immature stages

	SUSCEPTIBLES			RESISTANTS		
Species	3 Days	8 Days	16 Days	3 Days	8 Days	16 Days
O.s	1.6	1.6	1.6	156	110	110
S.o	498	498	498	574	574	574
R.d	156	70	30	574	574	574
C.f	33	12	12	498	498	498

Field trial data

Table 3 gives a summary of the cts obtained from trials A and B at different positions for 3, 8 and 16-day exposures. An extrapolation produced a 16-day estimate from the 13-day data from trial A. Because the dosage rate was different in the two trials, the cts from trial A were multiplied by a dosage correction factor as follows:

$$\text{Trial A} \times \frac{5.6\text{g/tonne}}{3.7\text{g/tonne}} \text{ or } \times 1.52$$

TABLE 3. Summary of field trial cts

	cts (gh/m^3)			
	Trial A		Trial B	
	Range	Mean (n=9)	Range	Mean (n=22)
3 days	5-86	26.4	15-492	93.5
8 days	15-206	66.9	78-880	325.9
16 days	27-298	103.2	91-964	371.8

DISCUSSION

There are clearly large differences in dosage required for the control of susceptible and resistant adults (Table 1). Table 2 shows the same trend for immature stages except for the naturally tolerant *S.oryzae*.

Table 3 clearly shows the benefit of dosing 43% of the aluminium phosphide in the ventilation ducting. Less gas is lost through the covering sheet and more is found at the bottom of the grain. The mean dosage-adjusted cts from trial A are 28%, 20% and 28% of the 3, 8 and 16 day cts respectively of those from trial B which indicates a large loss due to leakage in trial A.

The European Plant Protection Organisation (EPPO) fumigation standard

for phosphine (Anon, 1984) recommends exposure periods at 15°C as follows:-

	Days
O. surinamensis	3
C. ferrugineus	8
R. dominica	8
S. oryzae	16

A dosage of 1g of phosphine/m^3 equivalent to 1.4 g/tonne is suggested with an option to increase this level considerably for leaky situations. Trial B was dosed at 5.6 g/tonne.

An examination of the cts in Table 1 with those in Table 3 shows that susceptible adults would be controlled in both trials even in a 3-day exposure. However resistant adults would commonly survive an 8-day exposure. Trial B would produce better results for resistant adults though survival of *O. surinamensis*, *R. dominica* and *C. ferrugineus* may be expected if the exposure only lasted 8 days.

A comparison of the cts in tables 2 and 3 shows the situation is generally much worse for immature stages. The control of susceptible strains other than *Sitophilus* spp. would have been obtained in Trial B but not in Trial A. However all resistant strains would have survived a 16-day exposure in both trials except *O. surinamensis* in trial B.

The results have important implications for the current dosage schedule and indicate that it should be revised. In order to have a chance of controlling all stages of all resistant strains an exposure of 16 days at quite high dosages is indicated. Additional time must be allowed for diffusion from the site of generation. Friemel (1983) states that phosphine moves at 3m/day through grain. In addition to a longer exposure an increase in dosage rate to 10 g/tonne or higher may be required to cope with leaky situations. Dosing the ducting beneath bulks is beneficial though care should be taken not to exceed the explosive limit of 1.87% of phosphine in air within the ducts. This may require the ducts to be redosed at intervals yet to be determined.

An alternative dosing strategy is to introduce gas continuously. The evaluation is now underway of a mixture of 3% by weight of phosphine in liquid carbon dioxide applied from pressurised cylinders to ventilation ducts in bins and flat stores. This allows a continued controlled input of gas. However, the concentration does not build up to the localised high levels and hence high cts obtained when solid formulations are used. Toxicity studies are in progress with susceptible *S. oryzae* and resistant strains of all species to determine the exposure periods required at the lower concentration profiles obtained by this method.

ACKNOWLEDGEMENTS

Mr T.J. Wontner-Smith for assistance at trial B.
Dr C.H. Bell for discussions and comments on the manuscript.

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