SESSION 4C

RESISTANCE TO FUNGICIDES AND INSECTICIDES: RESULTS OF RECENT LABORATORY AND FIELD STUDIES

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FIRST OBSERVATIONS ON RESISTANCE IN VENTURIA INAEQUALIS AND GUIGNARDIA BIDWELLII TO ERGOSTEROL-BIOSYNTHESIS INHIBITORS IN FRANCE

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ABSTRACT

Isolates of V.inaequalis possessing different levels of resistance to fenarimol (LC_{90} up to 4.5 µg/ml) were obtained from an orchard in the South-West of France. Four of these isolates, when tested for cross-resistance to eight other EBIs, showed from low to high levels of resistance to most depending on the isolate and the fungicide. No cross-resistance was shown to fenpropimorph. Likewise, eight resistant strains of G.bidwellii, selected during in vitro fungitoxicity assays with an experimental triazole, possessed a considerable degree of cross-resistance to eight other EBIs. One strain was found to have a low level of resistance to fenpropimorph. Resistance in both the fungal pathogens seemed to be stable. When inoculated on young plants prior to curative treatments with fenarimol, one highly resistant strain of each of the two pathogens caused symptoms at doses normally inhibitory but the pathogenicity of the resistant form of G.bidwellii was considerably reduced. An urgent need to start monitoring programmes is emphasized.

INTRODUCTION

In recent years, ergosterol-biosynthesis inhibitors (EBI's) have shown great potential as disease control agents in agriculture. The majority of these fungicides (DMI's), despite their diverse chemical structures, have the same mechanism of action i.e. they interfere with ergosterol synthesis by inhibiting the C-14 demethylation step (de Waard & Fuchs 1982). In France, EBIs are now being widely used against diverse pathogens on various crops. On apple, these are used to control scab (Venturia inaequalis) and powdery mildew (Podosphaera leucotricha), while on grapes, their use is mainly against powdery mildew (Uncinula necator) and more recently against black-rot (Guignardia bidwellii). Normally on these hosts, EBIs are highly efficaceous at very low concentrations and exhibit remarkable post-infection curative activity (Olivier 1984).

Although laboratory mutants resistant to sterol-inhibiting fungicides are easily induced and show cross-resistance to other fungicides in this group, there are, as yet, only a few reports of naturally-occurring strains with reduced sensitivity to these fungicides (e.g. Schepers 1983 & 1985, Wolfe et al. 1984). Recently, Stanis and Jones (1985) have reported reduced sensitivity to some EBIs in field isolates of Venturia inaequalis obtained from an experimental orchard in the Federal Republic of Germany.

In view of the intensive use of various EBIs in apple orchards in France, scabbed leaf samples, obtained from different apple growing areas, were analysed in 1985 for the presence of strains of V.inaequalis, with reduced sensitivity. In addition, some isolates of Guignardia bidwellii from a culture collection were studied for their ability to develop resistance

in vitro. Cross-resistance studies were also carried out and the pathogenic behaviour of resistant strains of both pathogens was determined on young treated plants under glass-house conditions.

MATERIALS AND METHODS

V.inaequalis

Isolates

Leaf samples bearing sporulating lesions of V.inaequalis were procured in 1985 from some apple orchards which had received different treatments with EBIs. Conidial suspensions were plated on malt-agar (MA) medium (malt 15 g, agar 20 g, distilled water 1 l) amended with different concentrations (0 to 10 µg/ml) of fenarimol, a commonly used EBI. Sensitivity was assessed in terms of percent germination and germ-tube length. Selected conidia, bearing normal, elongated and bifurcated germ-tubes after 72 hrs of incubation on 1 µg/ml or more of fenarimol, were transferred to MA dishes to obtain monoconidial isolates thought to have reduced sensitivity. Sensitive forms were inhibited completely between 0,3 to 0,5 µg/ml and three of these were also isolated for comparison. Twenty-one isolates showing decreased sensitivity were further tested in the presence of different doses of fenarimol. On the basis of their different levels of resistance, four of these were selected for cross-resistance studies. These four resistant isolates, designated as BF1 to BF4, were obtained from an orchard near Balma (SW France) while two sensitive isolates designated M2 and M3, were from an orchard of Montreau (Central France) and one reference isolate (UZ4) came from St Pierre Montelimart (Western France).

Cross-resistance

The seven selected isolates (see above) were grown on MA for two weeks at 21 ± 1°C to provide inoculum. The nine EBIs used in this study were : triforine ("Saprol 19 EC", Sovilo), fenarimol ("Rubigan 4 EC", Pepro), nuarimol ("Trimidal 12 EC", Sandoz), prochloraz ("Sportak 45", Schering), triadimefon ("Bayleton 5 WP", Bayer), bitertanol ("Baycor 25 WP", Bayer), flusilazol ("Olymp 10 EC", Du Pont), penconazol ("Topaze 10 EC", Ciba Geigy) and fenpropimorph ("Corbel 75", Pepro). LC_{50} and LC_{90} values for inhibition of colony growth were determined using the poisoned-food technique. Petri dishes (90 mm diameter) containing MA, amended with a series of fungicide concentrations, were each inoculated with a 4.5 mm inoculum plug taken from the colony margin of a two-weeks old culture. Five dishes were inoculated for each treatment. After incubation for 3 weeks at 21 ± 1°C, colony diameter was recorded and percent inhibition of radial growth, compared to untreated control dishes, was calculated. Resistance factor (FR) was determined by dividing LC_{50} values of resistant strains with that of sensitive strains.

Glass-house test

One highly resistant isolate (BF1) and one sensitive reference isolate (UZ4) of V.inaequalis were inoculated onto two month old (4-leaf stage) seedlings of an apple hybrid of cvs. Golden x Richared (45 plants/ treatment). Conidial suspensions for inoculation (1.8×10^5 conidia/ml) were prepared from 12-day old cultures produced by plating conidia on MA plates. Inoculated plants were kept in a humidity control chamber maintaining 100 % R.H. for 68 hrs at 22 ± 1°C. This was followed by a spray to run-off with fenarimol at 10, 20, 40, 60 and 80 µg a.i./ml. Plants were air-dried for 2 hrs before being sprayed. Untreated, inoculated plants served as controls. A light, fine spray of water was given to the plants on the evening of the 8th & 9th days after inoculation and a plastic cover was provided during these two nights to facilitate the emergence of symptoms. Data on disease

intensity, were recorded for each treatment after 20 days of inoculation using a scale of 0-4 (0 = no visible symptoms, 4 = whole leaf surface sporulated).

G.bidwellii

Isolates, fungicides and cross-resistance

Five field isolates, designated B1, B2, B11, B12 and B16, were procured from the Plant Pathology Station of INRA, Bordeaux and were tested for their sensitivity in vitro to an experimental triazole. During observation on inhibition of colony growth, isolates B1, B2 and B11 produced small colony sectors on doses of this compound normally lethal. These sectors were transferred to plates of MA and tested further, using higher concentrations of the experimental triazole, to determine their resistance. Eight of the resistant strains, selected as having varying levels of resistance and designated as B2R1 to B2R5, B1aR1, B1aR2 and B11R1, were assayed, along with their sensitive parent isolates, for cross-resistance to nine other EBIs (the same as used for V.inaequalis). Inhibition of radial growth, compared to untreated checks, was recorded after three weeks incubation at 25°C. Resistant factors were calculated as described for V.inaequalis.

Glass-house test

An assay to determine the pathogenicity and resistance of one resistant strain (B2R1) of G.bidwellii, in comparison with one sensitive isolate (B2), was conducted on young grapevine plants (10 leaf stage) of cv. Muscadelle. Ten plants were used for each treatment. Plants were inoculated with pycniospore suspensions of each strain (1.5×10^5 pycniospores/ml) prepared from cultures grown on MA dishes for two weeks. Inoculated plants were provided with 100 % R.H. for 36 hrs, air-dried for 2 hrs and then sprayed with 6, 12 and 24 µg a.i./ml of fenarimol. After treatment, the plants were left in a glass-house having mean temp. of 25 ± 1°C during days and 12-16°C during nights. Disease development on leaves was recorded after 21 days of incubation by estimating the leaf surface area infected, using a scale from 0 to 10 (0 = no visible symptoms, 10 = whole leaf damaged).

RESULTS

In vitro sensitivity of isolates

V.inaequalis

During the first screening for resistance to fenarimol in field samples, twenty-one isolates, with varying levels of reduced sensitivity, were obtained from an orchard at Balma near Toulouse, whereas all the samples from other orchards yielded only sensitive isolates or isolates with slightly reduced sensitivity. While studying cross-resistance to other EBIs in four of the resistant isolates (BF1 to BF4), along with 3 sensitive isolates (UZ4, M2, M3), varying response to the nine test fungicides was observed (Table 1). The fenarimol-sensitive isolates showed high levels of sensitivity (mean LC_{50} 0,02 to 2,5 µg/ml) to all except triforine, which exhibited the lowest activity (mean LC_{50} 38 µg/ml). The four resistant isolates selected on fenarimol amended MA medium, exhibited positively correlated cross-resistance to all the eight efficaceous DMIs, but not triforine. Fenpropimorph showed a similar level of activity against both sensitive and resistant isolates.

To the majority of the test fungicides, isolate BF1 showed maximum resistance, followed by BF2. Isolates BF3 and BF4 appeared to be only moderately resistant. In general, resistance to fenarimol, penconazol and nuarimol was markedly greater (mean FR = 36 to 52) compared to the other fungicides used (mean FR = 7 to 19).

TABLE 1

LC_{50}, LC_{90} and factors of resistance (FR) of some sensitive and resistant isolates of V.inaequalis to various EBIs in vitro

Fungicides		Sensitive isolates				Resistant isolates				
		UZ4	M2	M3	Mean	BF1	BF2	BF3	BF4	Mean
Triforine	LC_{50} (µg/ml)	35	40	40	38.3	80	80	89	80	82.2
	LC_{90} (µg/ml)	100	90	100	96.6	100	100	100	100	100
	FR*	-	-	-	-	2	2	2.3	2	2
Fenarimol	LC_{50}	0.03	0.028	0.047	0.03	2.6	2	1.7	1	1.8
	LC_{90}	0.25	0.22	0.3	0.25	4.5	4.5	3.5	3.2	3.9
	FR	-	-	-	-	74.3	57.1	48.6	28.6	52.1
Nuarimol	LC_{50}	0.075	0.03	0.3	0.04	5	8	5	1	4.7
	LC_{90}	2	1	2.5	1.83	19	18.5	12.5	7.5	14.4
	FR	-	-	-	-	38.5	61.5	38.5	7.7	36.5
Pro-chloraz	LC_{50}	0.1	0.1	0.15	0.11	2	1	1	0.5	1.1
	LC_{90}	0.6	0.6	0.7	0.63	6	5.5	5.5	2	4.7
	FR	-	-	-	-	18.2	9	9	4.5	10.1
Tria-dimefon	LC_{50}	2	2	3.5	2.5	20	18.5	20	15	18.4
	LC_{90}	7	7	10	8	50	40	42.5	36.5	42.2
	FR	-	-	-	-	8	7.4	8	6	7.3
Biter-tanol	LC_{50}	0.4	0.4	0.5	0.43	7.5	5	2	2.5	4.2
	LC_{90}	2.5	2	3	2.5	25.5	21	16	8	17.6
	FR	-	-	-	-	17.4	11.6	4.6	5.8	9.8
Flusi-lazol	LC_{50}	0.02	0.02	0.25	0.09	0.3	0.6	0.4	0.3	0.4
	LC_{90}	0.25	0.1	0.3	0.21	4	5.5	1.5	1.5	3.1
	FR	-	-	-	-	14.3	28.6	19	14.3	19
Penco-nazol	LC_{50}	0.02	0.02	0.03	0.02	1.5	1.2	0.8	0.5	1
	LC_{90}	0.25	0.25	0.28	0.26	3.5	3.2	2.1	2	2.7
	FR	-	-	-	-	65.2	52.1	34.8	21.7	43.4
Fenpro-pimorph	LC_{50}	0.03	0.03	0.08	0.04	0.03	0.06	0.04	0.03	0.04
	LC_{90}	0.1	0.1	0.3	0.16	0.1	0.2	0.3	0.2	0.2
	FR	-	-	-	-	0.6	1.3	0.9	0.6	0.8

$$* = \frac{LC_{50} \text{ of resistant strain}}{LC_{50} \text{ of sensitive strains (Mean of UZ4, M2 \& M3)}}$$

TABLE 2

LC_{50} and factors of resistance (FR) of various sensitive and resistant isolates/strains of G.bidwellii to some EBIs in vitro

Fungicides		Sensitive isolates				Resistant strains								
		B1	B2	B11	Mean	B1aR1	B1aR2	B2R1	B2R2	B2R3	B2R4	B2R5	B11R1	Mean
Triforine	LC_{50} (g/ml)	12.4	15	16.4	14.6	45	45	50	45	45	45	40	40	43.75
	FR*	-	-	-	-	2.7	3	3.4	3	3	3	2.7	2.7	2.93
Fenarimol	LC_{50}	0.03	0.03	0.08	0.04	1	1.1	3.7	3.5	3.2	2.7	1.9	2.3	2.42
	FR	-	-	-	-	21.7	23.9	80.4	76	69.5	58.7	41.3	48.9	52.5
Nuarimol	LC_{50}	0.05	0.03	0.08	0.05	4.2	5	15.5	15	15.5	8.5	10	10.5	10.5
	FR	-	-	-	-	70	83.3	258.3	250	258.3	144.6	166.6	175	175.7
Pro-chloraz	LC_{50}	0.08	0.01	0.07	0.05	0.4	0.8	1.5	0.7	2	0.44	0.3	0.2	0.79
	FR	-	-	-	-	7.5	15	28.7	13.2	37.7	8.3	5.6	3.2	14.9
Tria-dimefon	LC_{50}	0.4	0.46	0.58	0.48	9.6	10	25.1	23.4	21.6	11.6	23.3	21.2	18.2
	FR	-	-	-	-	21.3	22.2	55.7	52	48	25.7	51.7	47.1	40.5
Biter-tanol	LC_{50}	0.29	0.1	0.2	0.19	0.28	0.38	5	4.5	3.8	3.2	3.15	1.45	2.7
	FR	-	-	-	-	1.47	2	26.3	23.7	20	16.8	16.6	7.6	14.3
Flusi-lazol	LC_{50}	0.03	0.01	0.03	0.02	0.24	0.3	4.8	3.9	5	1.5	1.5	1.8	2.4
	FR	-	-	-	-	12	15	240	195	250	75	75	90	119
Penco-nazol	LC_{50}	0.04	0.02	0.06	0.04	0.2	1.2	3.6	1.9	3.2	1.8	0.4	1.2	1.7
	FR	-	-	-	-	5.5	30	91.3	49	81	45	10	30	42.7
Fenpro-pimorph	LC_{50}	0.7	1.3	3.2	1.73	3.8	1.52	1.75	1.5	0.85	1.4	1.8	5	2.2
	FR	-	-	-	-	2.2	0.9	1	0.9	0.5	0.8	1	2.9	1.3

$$* = \frac{LC_{50} \text{ of resistant strain}}{LC_{50} \text{ of sensitive isolates (Mean of isolates B1. B2 \& B11)}}$$

G.bidwellii

The sensitive isolates were inhibited at very low doses of all EBIs used except triforine, which was only inhibitory at quite high doses. Almost all the resistant strains, selected in vitro during fungitoxicity assays of an experimental triazole, appeared to possess positively correlated cross-resistance to all the EBIs tested, except fenpropimorph. The degree of cross-resistance varied with the strain and nature of the fungicide tested (Table 2). For example in the case of nuarimol FR varied form 70 to 260 while in case of flusilazol FR's were between 12 and 250 depending on the strain. Generally, all the resistant strains showed greater level of resistance to nuarimol and flusilazol (mean FR = 175 and 119 respectively) than to other fungicides (mean FR = 15 to 50). In the case of triforine resistance was poorly marked. While most resistant strains were very sensitive to fenpropimorph, one strain (B11R1) seemed to have a low level of resistance to this fungicide.

Cultural characteristics of resistant strains

Resistant isolate BF1 of V.inaequalis showed a slightly lower growth rate than the sensitive isolates but had better sporulation. Other resistant isolates exhibited considerably inferior mycelial growth, as well as sporulation, in culture. There was little difference in colony colour between resistant and sensitive isolates.

All the resistant strains of G.bidwellii, except B2R3, showed reduced growth rates and sporulation compared to the sensitive, wild-type isolates. Strain B2R3 produced very few or no pycnidia on MA. Frequently strain B11R1 produced white sterile sectors in the colony. Colony colour was mostly black or dark brown except of B2R3 that had a light brown colour. Pycnidia were closely arranged in B2R4.

Glass-house tests

V.inaequalis

As evident from data in Table 3, sensitive isolate UZ4 did not produce symptoms on young plants treated with 10 µg/ml or more of fenarimol. In contrast, the resistant isolate BF1 caused infection even at 80 µg/ml of fenarimol, a dose twice the one recommended for use in orchards. However, disease severity was greatly reduced at 60 g/ml and above (about 50 % of the attack recorded at 10 or 20 µg/ml). Although isolate BF1 appeared to be as aggressive as the sensitive isolate UZ4, its incubation period was longer. The first symptoms appeared one day later with BF1 than with UZ4 in untreated plants (incubation period 10 days for UZ4). At 40 µg/ml, the symptoms were delayed by 3 days in BF1.

G.bidwellii

The sensitive isolate B2 could not produce symptoms at doses higher than 6 µg/ml of fenarimol (Table 4). At 6 µg/ml the disease intensity was quite low compared to the untreated control. In contrast, the resistant strain B2R1 caused considerable infection even at 24 µg/ml. However, B2R1 was notably less aggressive than its sensitive parent isolate (B2) as evident from the leaf-area attacked in untreated check plants (foliar attack less by 28 %). The time for first appearance of symptoms caused by the resistant strain was longer by two days, compared with that for the sensitive, where it was 12 days. The symptoms caused by B2R1 were first observed after 15 and 16 days following treatment with 12 and 24 µg/ml fenarimol respectively.

TABLE 3
Curative effect of fenarimol spays against leaf infections by sensitive (S) and resistant (R) isolates of V.inaequalis

Fenarimol (µg/ml)	% leaf area infected+		% efficacy/control	
	Isolates		Isolates	
	UZ4 (S)	BF1 (R)	UZ4 (S)	BF1 (R)
0 (control)	70	76	-	-
10	0	69	100	9.83
20	0	63	100	16.61
40 (*)	0	38	100	49.71
60	0	31	100	58.88
80	0	28	100	63.05

(*) dose recommended for use in the orchards.
+ figures are mean value for 180 (4 x 45) leaves.

TABLE 4
Efficacy of post-infection treatment with fenarimol against leaf infection by sensitive (S) and resistant (R) strains of G.bidwellii

Fenarimol (µg/ml)	% leaf area infected+		% efficacy/control	
	Isolates		Isolates	
	B2 (S)	B2R1 (R)	B2 (S)	B2R1 (R)
0 (control)	55	40	-	-
6	10	28	82	29.58
12	0	25	100	36.13
24	0	24	100	39.80

+ mean of 100 leaves

DISCUSSION

Isolates of V.inaequalis with varying levels of resistance to fenarimol were obtained from an orchard in South-West France which had received intensive treatment with EBIs for the past few years (Thind et al. 1986). Four of these isolates when tested against to eight other EBIs, showed cross-resistance to all except fenpropimorph, the levels of resistance depending on the isolate and fungicide used. During approx. nine months of in vitro fungitoxicity assays, the resistance seemed to be highly stable. Resistance factors for some of the present isolates were considerably higher than those observed by Stanis & Jones (1985) in isolates with decreased fenarimol sensitivity obtained from a West Germany orchard.

There is, as yet, no apparent failure of disease control by EBIs due to development of resistant strains of V.inaequalis, but the high pathogenicity of one resistant isolate tested in the present study shows that selection pressure on such strains may lead to the failure of control measures if prolonged use of these fungicides is continued. Such a situation has already been experienced with dodine and benomyl resistant strains of V.inaequalis (Szkolnik & Gilpatrick 1969, Wicks 1974). Isolation of fenarimol resistant

strains from the same orchard again in 1986 makes it evident that these strains may be fit to survive in nature.

The development of thirteen resistant strains of G.bidwellii, selected in vitro on a triazole amended nutrient medium, leads to the possibility of such forms occuring in nature also. The reduced pathogenicity of one resistant strain, in comparison with the sensitive, does not exclude the possibility of a higher pathogenicity in other resistant strains ; some sensitive natural isolates have also been observed to be less vigorous. The persistant nature of the resistance in these strains suggests that it is a genetically controlled character.

Appearance of fenarimol resistant forms of V.inaequalis in an orchard, as well as the selection of resistant strains of G.bidwellii in the laboratory, warns of a potential risk of disease control failure in practice as a result of increased use of the C-14 demethylation category of EBIs. The present findings emphasize the immediate need to start monitoring programmes for these two diseases and to work out a strategy to avoid this resistance problem in practice.

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FUNGICIDE RESISTANCE MONITORING AS AN AID TO TOMATO GREY MOULD MANAGEMENT[1]

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ABSTRACT

A simple rapid method for detection of benzimidazole or dicarboximide resistance in the tomato grey mould pathogen Botrytis cinerea Pers. was adopted by the extension service operating at Albenga (Northern Italy) in 1986. The technique is based on the percent germination of conidia on a simple medium amended with benomyl (5 mg/l) or vinclozolin (3 mg/l). Extension service was able to inform tomato growers within 24 hours of the fungicide resistance situation in their glasshouses. Spraying schedules were modified to avoid loss of control due to fungicide resistance.

INTRODUCTION

Surveys carried out on tomato crops grown under glasshouses in Northern Italy have shown a consistent presence of fungicide resistance in Botrytis cinerea Pers., causal agent of grey mould. Resistance appeared after a few years of repeated use of benzimidazoles (Porta-Puglia 1978) and dicarboximides (Gullino *et al.* 1981). While benzimidazole resistance is widespread, the incidence of dicarboximide resistance is variable (Gullino *et al.* 1984). Sometimes dicarboximide resistance developed in isolates of B. cinerea already resistant to benzimidazoles - double resistance (Gullino & Garibaldi 1986).

Many of the difficulties encountered in developing a disease management programme for grey mould in protected crops, where fungicide resistance is present, arise from the limited number of effective fungicides available and from the lack of information about the incidence of fungicide resistance in individual glasshouses within the same area. This second aspect is complicated in an agricultural system like that of protected crops in Italy, where many growers operate on a relatively small area, each following different spray schedules.

Monitoring methods for detecting antibiotic resistance in Erwinia amylovora and fungicide resistance in Monilia fructicola, M. laxa, Penicillium digitatum and P. italicum have been reviewed by Ogawa *et al.* (1983). A method for a simple, rapid detection of fungicide resistance in B. cinerea was developed for the extension service to determine the fungicide resistance situation in each glasshouse and to properly advise growers for effective fungicide use.

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MATERIALS AND METHODS

Development of sensitivity test

The following method was designed and tested in our laboratory during 1985. A simple medium (glucose 10 g/l, agar 20 g/l; Leroux & Gredt 1981), to which terramycin (50 g/l) had been added to reduce the growth of bacteria, was poured into 3 sector Petri plates. The sectors of each plate contained, respectively, unamended agar, agar plus benomyl (5 mg/l) and agar plus vinclozolin (3 mg/l). The fungicides were added to the molten medium, after autoclaving, as technical grade compounds from stock suspensions in methanol containing 100x the required concentration. The concentrations chosen are slightly higher than the respective Minimal Inhibitory Concentration, to detect low level resistant strains (Gullino *et al.* 1984).

Each glasshouse was sampled by collecting 8-10 fruit with sporulating lesions. Conidia from infected fruit were transferred by loop to test tubes containing 5 ml of distilled sterile water. After shaking, the conidial suspension (about $5x10^5$ conidia/ml) was streaked onto three plates (100 µl/ each sector). Plates were incubated at 25°C. After 16-18 hours, conidial germination was observed under a light microscope (100x). One hundred conidia/each sector were examined.

Typical spore response are illustrated in figure 1. In the presence of benomyl the benzimidazole sensitive conidia germinated, but further growth of germ tubes was inhibited (Fig. 1 a and c). Vinclozolin completely inhibited germination of dicarboximide sensitive conidia (Fig. 1 a and b). Percent inhibition is calculated from observation of 100 conidia from random field in each sector.

Surveys were carried out on tomatoes grown in Northern Italy (Albenga) and Sicily (Donnalucata). Infected fruit were sent to our laboratory and promptly tested.

Monitoring during 1986

During 1986, the monitoring technique was transferred to the extension service operating in the area of Albenga. The following supplies were prepared in our laboratory and provided to the technicians involved in the monitoring: three sector plates, each sector poured with the control (no fungicide), benomyl and vinclozolin medium, test tubes containing sterile water, sterile pipettes. Growers having problems in management of grey mould brought infected fruit directly to the extension service where isolation were carried out immediately. A single plate was used for each sample. Within 24 hours the growers were informed about the fungicide resistance situation in their own glasshouses and advised as to which fungicides they should avoid.

RESULTS AND DISCUSSION

The results obtained during 1985 revealed in Northern Italy that disease management is still possible with either dicarboximides and/or benzimidazoles (Table 1). In Sicily most glasshouses contained high percentage of strains of B. cinerea resistant to both fungicides (Table 2).

TABLE 1

Results of surveys carried out in 1985 on tomatoes grown in Northern Italy (Albenga)

Glasshouse	% of conidia resistant to	
	benzimidazoles	dicarboximides
1	73	0
2	80	1
3	n.t.*	84
4	n.t.	95
5	n.t.	0
6	n.t.	0
7	70	0
8	100	27
9	0	0
10	75	0
11	80	77
12	n.t.	29
13	60	30
14	0	0
15	100	20
16	89	0
17	100	9
18	46	0
19	100	0
20	100	0
21	0	0
22	100	81
23	70	0
24	87	0
25	17	0
26	0	0
27	0	0

* n.t. = not tested

For the investigation we chose to sample from two contrasting situations. The area of Albenga had an effective extension service with good disease management, but Sicily was under severe disease pressure and with little advices for disease management.

The monitoring carried out in 1986 by the extension service (Table 3) revealed several locations where alternate programmes were recommended. No practical problems were encountered by the technicians applying the method. Advice on future disease management in a particular glasshouse in Northern Italy depended upon which of the four possible patterns of fungicide

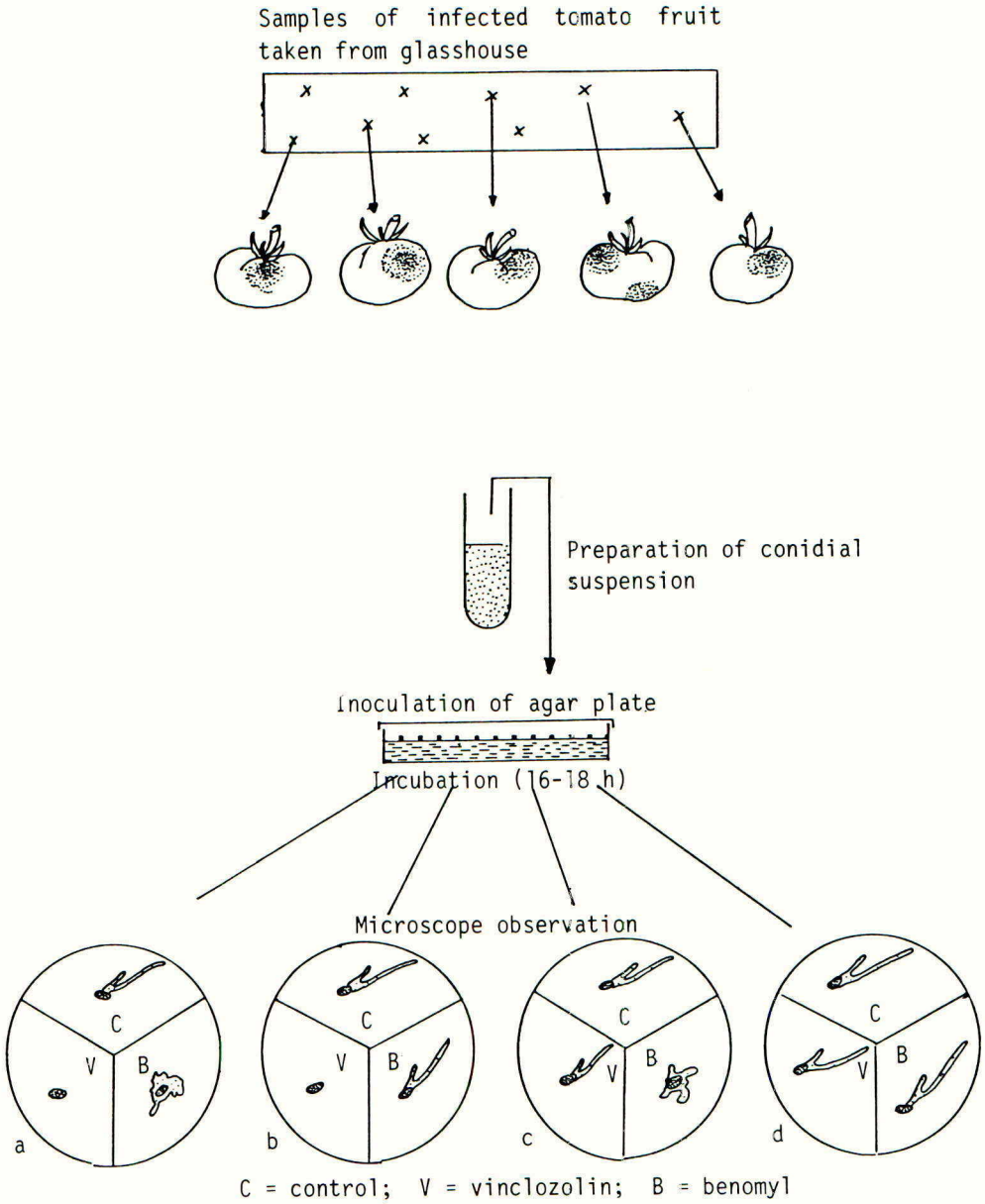

Fig. 1. Scheme of operations carried out for monitoring fungicide resistance in B. cinerea and possible resistance patterns.

resistance was shown in the laboratory test on the sample of B. cinerea taken from the glasshouse (Fig.1). When population of B. cinerea is:

a) sensitive to benzimidazoles and dicarboximides: a moderate use of benzimidazoles (1-2 sprays/season) and dicarboximides (2-3 sprays/ /season) is advised. If many treatments are required, the alternation of other fungicides (chlorothalonil, captafol, captan, dichlofluanid, folpet) is recommended;

b) resistant to benzimidazoles and sensitive to dicarboximides: if 20% or more of conidia germinate on benomyl amended media, the use of benzimidazoles is discouraged. Dicarboximides are recommended in alternation with other fungicides (see a);

c) sensitive to benzimidazoles and resistant to dicarboximides (situation never encountered in practice): the use of dicarboximides should be discontinued. Benzimidazoles could be sprayed in alternation with other fungicides;

d) resistant to both benzimidazoles and dicarboximides: if the frequency of resistance is still low (<20% of germinating conidia) one spray/ season of each group of fungicides may still be effective. Other chemicals must be alternated. When the frequency of resistant conidia is >20%, fungicides at risk should be discontinued. Monitoring regularly carried out will show when the pathogen population shifts to >80% sensitivity and careful reintroduction of fungicides at risk will be reconsidered.

TABLE 2

Results of surveys carried out in 1985 on tomatoes grown in Sicily

Glasshouse	% of conidia resistant to	
	benzimidazoles	dicarboximides
1	100	99
2	99	98
3	80	70
4	100	100
5	100	96
6	100	96
7	100	97
8	100	96
9	100	98

Experience in Northern Italy and Sicily demonstrates that monitoring and fungicide management can help to avoid fungicide failure due to resistance.

TABLE 3

Results of surveys carried out in 1986 on tomato grown in Northern Italy (Albenga)

Glasshouse	% of conidia resistant to	
	benzimidazoles	dicarboximides
1	70	30
2	70	10
3	80	40
4	50	80
5	20	40
6	10	0
7	15	5
8	20	20
9	80	50
10	80	0
11	70	70
12	25	25

The technique described above can also be applied to different crops. It is accurate, rapid and it detects low levels of fungicide resistance. This technique can be applied by extension service thus reducing problems caused by storage and shipping of samples. It requires very simple equiment (a microscope, plates, tubes, pipettes, a simple medium).

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ACYLALANINE RESISTANCE IN PHYTOPHTHORA INFESTANS IN NORTHERN IRELAND

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ABSTRACT

Between 1981 and 1985, in annual surveys of the incidence of acylalanine-resistant Phytophthora infestans on commercial potato crops, the proportion of resistant isolates varied from year to year between 9 and 49% and no trend was observed. Resistant isolates were obtained from both acylalanine-treated crops and those sprayed with protectants only, but most originated from the regions of greatest acylalanine usage. Resistance was not related to poor disease control. In a field trial, inoculated with resistant and sensitive isolates of P. infestans, formulations containing an acylalanine plus mancozeb performed as well as or better than mancozeb alone.

INTRODUCTION

Fungicides of the phenylamide group, generally referred to as acylalanines, are very active against Oomycete fungi. However, in many cases, when they have been used intensively to control diseases of agricultural or horticultural crops, acylalanine-resistant strains of pathogens have emerged (Davidse 1982).

In 1978, a formulation containing the acylalanine metalaxyl plus the non-systemic mancozeb ('Fubol 58WP', Ciba-Geigy) was introduced onto the UK market for the control of potato blight (caused by Phytophthora infestans). This product was used in Northern Ireland from 1978 to 1980 and performed well. However, during 1980 in the Netherlands and the Republic of Ireland, where metalaxyl alone was used for blight control, disease control failed due to selection of acylalanine-resistant strains of P. infestans (Davidse et al 1981; Dowley & O'Sullivan 1981). Despite the lack of any deterioration in control with metalaxyl plus mancozeb in N. Ireland, tests on isolates of P. infestans from tubers of the 1980 crop revealed the presence of acylalanine-resistant strains (Cooke 1981). Concern that further selection pressure in favour of resistance would result in the performance of metalaxyl plus mancozeb being impaired resulted in growers being advised by the Department of Agriculture for N. Ireland (D.A.N.I.) not to use this or related products in the period 1981-1984. This paper reports the results of an annual survey of the occurrence of acylalanine-resistant P. infestans during 1981-5, and the pattern of usage of acylalanine-containing products. A field trial to investigate the effectiveness of acylalanine-containing formulations in the presence of resistant P. infestans is described.

MATERIALS AND METHODS

Sampling of potato crops

It was considered impractical to attempt to achieve sampling which was completely random or strictly comparable between seasons, since the incidence of potato blight varies greatly from year to year and potato crops are subject to a statutory minimum five year rotation in N. Ireland. The twenty-one Potato Inspectors, who are each responsible

for a specific geographical area of the potato-growing regions of N. Ireland, were therefore asked to supply four to six samples of blight-infected foliage collected from separate crops within their areas. Where possible, inspectors were asked to sample one true leaf from five sites within the crop, the leaves from each crop being bulked together. Data on potato cultivar, fungicide usage and blight incidence were collected for each sampled crop. In 1985, collation of data was facilitated by the use of a DATATRIEVE program, which allowed rapid sorting and extraction of results. At the end of the 1983-5 seasons, the inspectors were asked to estimate the percentage of the seed potato crop in their areas treated with acylalanine-containing products and in 1985 also to supply details of product usage and disease incidence.

Maintenance of isolates

On receipt, samples were placed in humid boxes overnight to encourage sporulation. Sporangial suspensions were prepared by using a sterile brush to transfer sporangia to cold, distilled water. Zoospore release was stimulated by chilling (5° C, 2-3 h) and then allowing the suspension to warm to room temperature before inoculation. The suspensions were used to inoculate detached leaflets, taken from glasshouse-grown plants (cv King Edward), contained in humid boxes, and to test for acylalanine-resistance. Isolates were transferred to fresh potato leaflets at 7-10 day intervals until test results were confirmed.

Tests for acylalanine-resistance

During 1982 and 1983, detached potato leaflets were dipped in metalaxyl solutions (100 or 2 mg/l) or water, inoculated with the test isolate and assessed after five and seven days' incubation for the presence of sporulation (Cooke 1981). Using this method, it was occasionally observed that, after prolonged incubation, a limited amount of growth occurred on metalaxyl-treated leaflets even with isolates of *P. infestans* never previously exposed to the fungicide. For this reason, from 1984 onwards a modification of the method described by Carter *et al* (1982) was used.

Discs (10 mm diameter) cut from leaves of glasshouse grown potato plants cv King Edward were floated, abaxial surface up, on solutions of metalaxyl (100 or 2 mg/l in 0.1% vol/vol acetone/water) or acetone/water alone. The discs (six per treatment) were inoculated with drops (20 µl) of a sporangial/zoospore suspension (c 10^5 sporangia/ml) of the appropriate test isolate. Sporulation was assessed after seven and 10 days' incubation (15° C, daylight). Isolates were designated acylalanine-resistant if they sporulated on the 100 mg/l metalaxyl-treated discs and sensitive if growth occurred on at least four out of six untreated, but on no metalaxyl-treated, discs. Isolates which grew on less than four control discs were re-tested and all resistant isolates were tested at least twice. Standard resistant and sensitive isolates were included in all tests.

Field trial, 1984

Potato tubers cv King Edward SE1 were planted (1 May 1984) at Newforge, Belfast in fully randomised blocks using 5 replicate plots per treatment. Each plot (2.8 x 3.0 m^2) contained four rows of ten tubers. Pairs of rows of unsprayed potato plants adjacent to each treated plot were inoculated (5 July) and served as an infection source. In these rows, every fourth plant was inoculated, the plants receiving alternately

either an acylalanine-resistant or sensitive strain. Treatments (Table 4) were applied at manufacturers' recommended rates in c. 560 l/ha using a Cooper, Pegler CP3 knapsack sprayer (hollow conespray nozzle, pressure c. 300 kPa, flow rate 720 ml/min). The plots received five applications (4, 18 July and 1, 8, 15 August) and infection was then allowed to develop until plants were killed. Foliage blight incidence was assessed using the British Mycological Society key (Anon 1947) with the addition of a 0.01% category. Samples of tubers (250 per plot) were stored for 8 weeks after lifting, then washed and assessed for tuber blight.

RESULTS

Usage of acylalanine-containing products

During the period 1981-4, DANI, in cooperation with the manufacturers of acylalanine-containing products, did not recommend their use in N. Ireland. However, as these products were freely available in Great Britain, many growers who had obtained excellent disease control with them in 1978-1980 continued to use them. This occurred particularly in Counties Antrim, Londonderry and Tyrone, where the cool, damp climate favours blight and where, consequently, many growers had been quick to appreciate the value of acylalanines (Table 1). In County Down, where

TABLE 1

Estimated proportion of seed potato crop area treated with acylalanines

Year	Treated seed crop area (%)			
	Antrim	Down	Londonderry	Tyrone
1983	50	10	20	30
1984	50	10	30	20
1985	70	30	60	50

blight control had generally been less of a problem, most growers continued to use protectant products. The data in Table 1 refer only to seed crops, since accurate data for the ware crops were difficult to obtain, these crops not being regularly inspected. It is probable that the proportion of ware crops treated with acylalanines was similar or a little lower than for the seed crops. The marked increase in crop areas treated in 1985 reflects the interaction of two factors, the re-introduction of acylalanine-containing products on to the N. Ireland market with manufacturers' recommendations and the extremely wet season which favoured rapid spread of blight. Of the products containing acylalanines recommended for the control of potato blight, the one in most widespread use in N. Ireland between 1982 and 1985 was metalaxyl + mancozeb with some usage of cyprofuram + mancozeb ('Stanza', FBC), ofurace + manganese zinc ethylenebis(dithiocarbamate) ('Patafol Plus', ICI Plant Protection) and, in 1985, oxadixyl + mancozeb ('Recoil', Bayer).

Incidence of acylalanine-resistant P. infestans

The results of the annual surveys, including the 1981 figures for comparison, are shown in Table 2. The sources of the 1985 isolates are depicted in Fig 1. The number of viable samples received each season depended on blight incidence which was influenced by the weather conditions (Table 3). In 1982 and 1983, the warm, dry summer weather resulted in few cases of blight, while in 1984 a period of high rainfall in August increased the amount of infection. In the exceptionally wet 1985 season, blight was present on crops in all areas of N. Ireland and it was possible to obtain as many samples as could be tested in the time available.

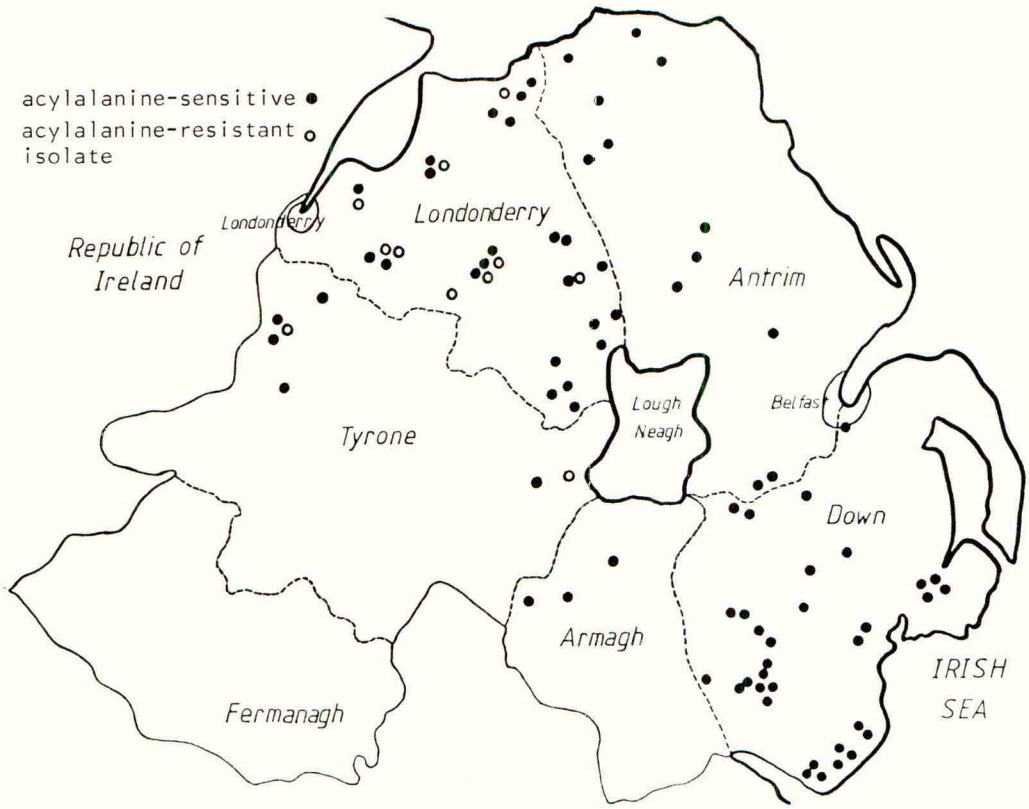

Fig. 1. Sources of isolates of Phytophthora infestans tested for acylalanine-resistance in 1985.

TABLE 2

The incidence of acylalanine-resistant P. infestans, 1981-5

Year	Number of resistant isolates[a]					Proportion resistant (%)
	Antrim	Down[b]	Londonderry	Tyrone	Total	
1981	2(15)	1(32)	7(25)	0(6)	10(78)	12.8
1982	1(3)	3(15)	1(10)	2(3)	7(31)	22.6
1983	1(9)	0(12)	0(5)	2(6)	3(32)	9.4
1984	4(9)	4(15)	13(19)	3(6)	24(49)	49.0
1985	0(12)	0(36)	9(32)	2(7)	11(87)	12.6

a total numbers of viable isolates tested are given in brackets
b including Co. Armagh

TABLE 3

Meteorological data collected at Newforge Lane, Belfast

Year	Three monthly rainfall totals (mm)[a]			
	January-March	April-June	July-September	October-December
1982	234(3.3; 9.7)	212(7.0; 15.5)	197(10.6; 18.5)	387(4.0; 10.2)
1983	232(2.9; 8.3)	171(6.3; 13.6)	133(11.7; 19.7)	253(5.7; 11.4)
1984	385(1.8; 7.5)	93(7.0; 15.5)	233(10.9; 18.9)	275(4.5; 10.7)
1985	205(1.0; 6.7)	190(6.5; 14.2)	342(10.8; 17.2)	176(4.2; 10.2)

a mean daily minimum and maximum temperatures (°C) are given in brackets

The proportion of resistant isolates fluctuated from year to year and no consistent trend was observed. The proportion of resistance tended to be greatest in Counties Londonderry and Tyrone, where there was much usage of acylalanine-containing products (Table 2). In each season, the great majority of isolates was obtained from crops which had been sprayed with protectant fungicides only. This was not only due to the pattern of product usage, but also to the good disease control achieved on crops sprayed with acylalanine + protectant products, which made it difficult to obtain blight samples from these crops, particularly in 1982-84. In 1985, 65 isolates were obtained from protectant-sprayed crops and 22 from crops treated with acylalanines; of these, three of the former (4.6%) and eight of the latter (36.4%) were resistant.

A wide range of cultivars was sampled each season. Thus in 1985, isolates were obtained from crops representing 18 cultivars, the greatest

numbers coming from cvs King Edward (17) and Arran Banner (14). The resistant isolates originated from crops of cvs King Edward (7), Kerr's Pink (2) and Up-to-date (2). These three cultivars are all very susceptible to blight and are mainly grown in the north-west of the Province where acylalanines are extensively used.

In 1982-4, the dry weather resulted in good blight control in most crops. In 1985, much more infection developed, as conditions both favoured the disease and were unsuitable for fungicide application for long periods. However, the crops yielding resistant isolates were not more severely infected than others. The greatest losses from blight occurred in Co Down on protectant-sprayed crops infected with sensitive strains of P. infestans, whereas in Co Londonderry where the incidence of resistance and acylalanine usage was greater, disease control was much better.

Field trial, 1984

Dry weather in July and early August caused a slow spread of infection after inoculation, but when heavy rainfall occurred in late August disease development suddenly accelerated. On 22 August there were no significant differences between treatments and only the mancozeb sprayed plots had more than 1% infection, but by 4 September all plots were severely infected (Table 4). On 4 and 12 September, plots sprayed

TABLE 4

Performance of fungicides in controlling potato blight in a field trial

Treatment		Foliage blight infection (%)		Tuber blight (%)[a]	Mean yield per plot (kg)[b]
acylalanine; g/ha	+ mancozeb (g/ha)	4 Sept	12 Sept		
none	1280	51.0	65.3	12.1	50.4
cyprofuram; 200	+ 1400	40.0	63.4	10.0	50.3
metalaxyl; 150	+ 720	54.0	72.0	8.0	46.5
ofurace; 114	+ 1340	43.5	63.8	10.4	49.0
oxadixyl; 200	+ 1400	40.6	59.0	7.1	54.1
LSD ($p<0.05$)		7.79	6.87	3.70	3.27

a data are arcsin transformations
b excluding tubers less than 30 mm diameter

with cyprofuram or oxadixyl both plus mancozeb were significantly less severely infected than those sprayed with metalaxyl + mancozeb or mancozeb alone. However, on 21 September, no treatment was significantly different from mancozeb alone. Only the plots which received sprays containing metalaxyl or oxadixyl yielded tubers with significantly less blight infection than those from the mancozeb-sprayed plots.

DISCUSSION

When acylalanine-resistance was first identified in P. infestans in N. Ireland, it was considered likely that the metalaxyl + mancozeb formulation would not continue to give adequate disease control, since, at the recommended rate, it gives only 720 g mancozeb/ha compared with 1360 g/ha for products containing mancozeb alone (Cooke 1981). To date, this has not proved to be the case in practice in N. Ireland, nor in the West of Scotland (Holmes 1984). In contrast, Davidse et al (1981) reported disease control failures on metalaxyl + mancozeb sprayed crops in the Netherlands in 1980, and showed poor disease control in field trials with this formulation on cv Bintje (Davidse et al 1983). In the field trial reported here, and in other trials in 1983 and 1985 (L R Cooke, unpublished), where plots have been exposed to acylalanine-resistant inoculum, metalaxyl + mancozeb has never given significantly poorer foliage blight control than mancozeb alone and has given better tuber blight control. With acylalanine formulations which gave a greater rate of mancozeb, foliage blight control was generally significantly better than with mancozeb alone. The effect of the rate of mancozeb used in the various acylalanine formulations is difficult to evaluate, since the acylalanines themselves differ in inherent activity against P. infestans (Kendall & Carter 1984), persistence (Cooke 1986a) and possibly in secondary modes of action (Fisher & Hayes 1985). However, it appears that when much acylalanine-resistant P. infestans is present, formulations with a "full-rate" of mancozeb give the best foliage blight control (although not necessarily the best tuber blight control), but that in all the formulations the acylalanine component contributes to the overall disease control.

On commercial crops, where most infections were caused by acylalanine-sensitive P. infestans, the contribution of the acylalanines to disease control was more marked. Thus, in 1985 when about half the seed crops were treated with acylalanines, most cases of severe blight infection occurred in crops sprayed with protectant fungicides alone (Cooke 1986b) and were not associated with the occurrence of resistance.

This situation is in marked contrast to that of the control of Bremia lactucae on lettuce with acylalanine + mancozeb formulations, where the build-up of resistance led to reduced disease control (Norman 1984).

The fluctuations from year to year in the proportion of crops from which acylalanine-resistant P. infestans could be isolated do not appear to be related to climatic conditions or disease incidence and the possibility cannot be excluded that they are an artefact of the sampling method. Considering the continued usage of acylalanine-containing products in N. Ireland and the apparent fitness of the resistant isolates in field trials and on commercial crops, as demonstrated by the number isolated from protectant-sprayed potatoes, it is surprising that the resistant strains of P. infestans have not so far dominated the population. Resistant isolates have been obtained from tubers of acylalanine-treated crops, but levels of infection have generally been very low, so it is possible that relatively few resistant strains manage to over-winter. Further studies are needed to investigate this possibility.

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A COMMERCIAL APPROACH TO DELAYING OR PREVENTING THE BUILD-UP OF CEREAL DISEASE RESISTANCE

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ABSTRACT

Fungicides from different groups (ie with different modes of action) were evaluated, in mixtures and in alternation for control of cereal diseases. Mixtures containing both flutriafol (group 2) and ethirimol (group 4) applied as seed treatments, were superior to a single triazole (group 2) treatment for control of barley powdery mildew. Fenpropidin and fenpropimorph (group 3) were superior to propiconazole (group 2) as foliar sprays for mildew control but lacked the activity of the latter against some other barley diseases, such as net blotch. Mixtures of flutriafol with captafol (group 8) or chlorothalonil (group 9) gave high levels of Septoria control on wheat. Alternating the use of compounds from different groups in programmes against foliar disease on winter and spring barley provided superior control to a programme based on triazoles only. Improved disease control resulted in improved final yields.

INTRODUCTION

A range of chemicals is now available for cereal disease control and these have been classified into numbered groups according to their modes of action (Anon, 1986).

Fungicide resistance is encouraged by the constant use of compounds from the same group. Members of a group have a common mode of action and cross-resistance between them is normal. Alternating the use of fungicides from different groups in a programmed approach to disease control is recognised as one way of delaying or preventing the development of resistant strains of pathogens. Another means generally recommended is the use of mixtures of fungicides, having differing modes of action against the pathogen.

Chemicals from several groups have been tested in recent ICI trials programmes, both alone and in mixture, as part of a programmed approach to season-long disease control. The objective of these trials has been to prove that cereal diseases may be well-controlled by alternating or mixing during a season, compounds having different modes of action.

The trials described in this paper included commercial formulations, and tank mixes, of the compounds listed below:-

Group	Compounds
1	carbendazim, thiabendazole, fuberidazole
2	flutriafol, propiconazole, triadimefon, triadimenol, prochloraz
3	fenpropidin, fenpropimorph
4	ethirimol
9	captafol
10	chlorothalonil
12	organomercury seed treatments

MATERIALS AND METHODS

A range of seed treatments and foliar sprays was tested either as pre-formulated mixtures or as tank mixes. Table 1 lists the treatments used, the fungicide group to which each component belongs and application rates.

TABLE 1

Compounds tested, their fungicidal groups and their application rates.

Active Ingredients	Fungicide Groups represented	Rates
Seed treatments:		ppm on seed
Flutriafol/ethirimol/thiabendazole ('Ferrax')	2/4/1	150/2000/5
Triadimenol/fuberidazole ('Baytan')	2/1	375/45
Ethirimol ('Milstem')	4	3886
Foliar sprays:		g(ai)/ha
Fenpropidin ('Patrol')	3	750
Fenpropimorph ('Corbel')	3	750
Propiconazole ('Radar')	2	125
Prochloraz ('Sportak')	2	400
Triadimenol ('Bayfidan')	2	125
Chlorothalonil ('Bravo' 500)	10	1000
Carbendazim ('Stempor' DG)	1	250
Flutriafol/chlorothalonil	2/10	94/750
Flutriafol/captafol ('Impact' Extra)	2/9	94/750
Triadimefon/captafol ('Bayleton' CF)	2/9	125/1300
Flutriafol/carbendazim (Early 'Impact')	2/1	118/188
Triadimefon/carbendazim ('Bayleton' BM)	2/1	125/250

All trials were fully replicated using plots arranged in randomised block designs and were conducted throughout the main cereal growing areas of the UK. Foliar sprays were applied using CO_2 knapsack sprayers in 200-250 l/ha of water. Seed treatments were applied using a 'Rotostat' machine.

Foliar disease was assessed by eye as percentage leaf area affected individually on 20 leaves per plot while yields were determined for individual plots using a plot combine harvester. Grain yields were corrected to a moisture content of 15%.

RESULTS AND DISCUSSION

Results of trials are presented in Tables 2 to 9. Where data from single trials are presented, they are analysed statistically using Duncan's multiple range test (Duncan, 1955), values within that trial followed by a common letter are not significantly different at P = 0.05.

The timings of spray applications were related to the stage of development of the crop, expressed on the decimal scale (Zadoks et al., 1974). The efficacy of treatments is expressed as % reduction compared to the untreated, calculated using the equation $\frac{C-T}{T} \times 100$ where C = untreated value and T = treated value. Yields from treated plots are given as % of those from untreated plots. Yields from untreated plots and disease levels on untreated plots are actual values.

Seed treatment trials from 1984 to 1986 compared the mildew control from seed treatments containing a single mildew fungicide from group 2 (triadimenol) with that of a mixture of two compounds from groups 2 (flutriafol) and 4 (ethirimol). Additionally, in 1984, ethirimol alone was included. Table 2 presents the results from six trials which demonstrated the superior performance of the mixture compared with the triadimenol-based formulation. The flutriafol/ethirimol/thiabendazole formulation consistently gave superior control throughout this period, a time when mildew populations had become less sensitive to triazole fungicides used alone (Heaney et al., 1984).

TABLE 2

Control of mildew[(a)] of spring barley with seed treatments.

Year	1984		1985		1986	
Treatment / Trial No.	EA12	NA21	EA17	SW19	17/1	11/1
Wks after drilling	13	13	13	12	8	12
Untreated (actual)	(16.7)a	(10.4)a	(45.9)a	(11.6)a	(13.6)a	(4.8)a
Flutriafol/ethirimol/ thiabendazole	93c	99c	92b	80b	79b	83b
Triadimenol/fuberidazole	56b	78b	42c	57c	63c	65c
Ethirimol (+ Organomercurial)	85c	97c	nd	nd	nd	nd
Leaf assessed:	L3	L4	L3	L2	L4	L3
Cultivar:	Georgie	Koru	Gold-marker	Triumph	Triumph	Triumph

nd = no data; this treatment not included in trial.
(a)= figures are % reduction in disease - see under Results and Discussion

The superior control of mildew was reflected in significant yield improvements in three out of four trials in 1984/85 compared with yields obtained following treatment with the triazole-only formulation (Table 3). Yield data for 1986 are not yet available.

TABLE 3

Yields[a] from spring barley treated with seed treatments.

Treatment	Year	1984		1985	
	Trial No.	EA12	NA21	EA17	SW19
Untreated (t/ha)		(4.95)a	(5.44)a	(4.45)a	(5.13)a
Flutriafol/ethirimol/thiabendazole		126c	115b	109b	127b
Triadimenol/fuberidazole		110b	110b	101c	111c
Ethirimol (+ Organomercurial)		122c	114b		

(a)= as % of untreated.

Ethirimol sensitivity in barley powdery mildew declined during the period of widespread use after 1969 (Shephard et al., 1985) but increased again as its use declined following the introduction in the late 1970's of the triadimenol/ fuberidazole seed treatment and other triazole-based fungicides (Heaney et al., 1984). It is hoped that the mixture of flutriafol with ethirimol will continue to provide effective mildew control by delaying or preventing the development of pathogen strains with reduced sensitivity to both groups of fungicides.

The reduced efficacy of triazole (group 2) fungicides against mildew in recent years has resulted in the more widespread use of morpholines and a piperidine (group 3) for improved control of mildew. Table 4 presents results from 3 Scottish trials in 1984, using foliar sprays at Zadoks 31, where fenpropidin was compared with propiconazole and fenpropimorph. In these trials, seed was treated with an organomercurial treatment only. The fenpropidin and fenpropimorph treatments gave superior control of mildew on spring barley compared with that from the group 2 (propiconazole) treatment. Nevertheless, the triazole-based treatments continued to provide control. Final yields from the 3 trials were substantially improved by all three treatments.

TABLE 4

Mildew control and yield increase in spring barley trials (1984)* following application of a single spray at Zadoks 31.

Treatment	% Mildew control[a]			Yield[b]
Weeks after treatment (WAT)	2	4	6	
Untreated (actual)	(12.0)	(21.0)	(44.0)	(5.31 t/ha)
Fenpropidin	96	85	73	120
Propiconazole	88	73	47	109
Fenpropimorph	96	85	78	120
Leaf assessed:	Means of L2 and L3			
Cultivar:	Golden Promise			
Mean disease pre-spray:	11.0% L3			

*= mean of 3 trials (a)= see Table 2 (b)= as % of untreated

That treatments with propiconazole continued to produce significant yield increases is explained partly by the good control of mildew despite the decrease in sensitivity of mildew populations to triazoles. Also of significance, however, is that some of the group 2 fungicides have a broader spectrum of activity than group 3 compounds and control other diseases which affect yield. Table 5 presents results obtained in 1983 and 1984 against Rhynchosporium leaf blotch (Rhynchosporium secalis) on winter barley.

TABLE 5

Control of leaf blotch on winter barley with foliar sprays at Zadoks 31.

Treatment	Trial No	SW16		EM17			SW25	
	WAT	4	6	2	6	2	4	6
Untreated (actual)		(18.6)a	(6.5)a*	(5.8)a	(2.5)a	(9.9)a	(14.1)a	(10.8)a
Fenpropidin		nd	nd	nd	nd	88b	71b	77b
Propiconazole		90c	71b	74b	98b	96b	97c	98b
Prochloraz		86c	59b	76b	100b	nd	nd	nd
Fenpropimorph		nd	nd	nd	nd	88b	87bc	83b
Leaf assessed:		L1	*	L2	L1	L3	L2	F1
Cultivar:		Igri		Igri			Igri	

nd = no data; this treatment not included in trial
* = disease levels assessed on a 0 (no disease) - 10 (severe disease) scale for the whole plant

Both the group 2 compounds (propiconazole and prochloraz) and the group 3 compounds (fenpropidin and fenpropimorph) controlled leaf blotch effectively under the low disease pressure but propiconazole was a better treatment compared with the group 3 compounds. Against net blotch (Pyrenophora teres) on winter barley (Table 6) the superior activity of propiconazole compared to fenpropidin and fenpropimorph was clearly evident.

TABLE 6

Control of net blotch[(a)] in winter barley with a single spray at Zadoks 57.

Treatment	Trial No	1	2	3
	WAT	4	4	4
Untreated (actual)		(5)a	(11)a	(68)a
Propiconazole		60b	72b	44b
Fenpropimorph		1a	18a	9a
Fenpropidin		9a	21a	0a
Leaf assessed:		L2	L2	L2
Cultivar:		Igri	Gerbel	Sonja

(a)= see Table 2

These results demonstrate the value of selecting the fungicide according to the disease risk. For example, selection between propiconazole and fenpropidin treatment of winter barley depends on the main disease targets. Where mildew is the main target then fenpropidin would be the preferred compound; where leaf blotch, net blotch or rusts are more important then propiconazole would be chosen.

In addition to its role in delaying or reducing the manifestation of disease resistance, mixing of fungicides allows their individual biological properties to be used in combination. Table 7 presents the results from 3 trials where combinations of triazoles with residual protectant compounds (captafol from group 9 or chlorothalonil from group 10) were tested against Septoria leaf spot of winter wheat.

TABLE 7

Control of Septoria leaf spot, winter wheat with a single spray at Zadoks 39.

Treatment	Trial no WAT	A1 4	A5 6	A7 4
Untreated (actual)		(36.7)d	(68.6)d	(19.3)c
Flutriafol/Chlorothalonil		81.5ab	71.4ab	44.0a
Flutriafol/Captafol		83.1ab	73.2ab	52.3a
Triadimefon/Captafol		73.3bc	62.7b	42.0ab
Chlorothalonil		68.9c	30.9c	8.8bc
Leaf assessed:		L3	L3	L4
Cultivar:		Longbow	Norman	Longbow

Chlorothalonil used alone gave significant control of the disease in two trials but a mixture of flutriafol and chlorothalonil was significantly superior due to the systemic activity of the triazole. Mixtures of flutriafol with captafol or chlorothalonil have given consistently higher levels of Septoria control than that of triazoles, captafol or chlorothalonil used alone.

The value of mixtures and alternating the use of fungicides from different groups, may be seen in Tables 8 and 9.

TABLE 8

Control of mildew[(a)] and yield increase[(b)] in 1985 with different spring barley programmes.

Seed Treatment	Foliar spray at GS37-49	Trial 1 Somerset Disease Control 14 WAD* 2 WAT+	Trial 1 Somerset Yield	Trial 2 Suffolk Disease Control 15 WAD 2 WAT	Trial 2 Suffolk Yield	Trial 3 Warwicks Disease Control 18 WAD 4 WAT	Trial 3 Warwicks Yield
Flutriafol/ ethirimol/TBZ	None	95cd	127c	86c	109b	68b	106b
Flutriafol/ ethirimol/TBZ	P'conazole	98d	136d	96d	122c	96c	115c
Triadimenol/ fuberidazole	None	82bc	111b	50b	101a	49b	100a
Triadimenol/ fuberidazole	Triadimenol	66b	119b	92c	106b	61b	106b
Untreated (actual % disease)		(10)a	(5.13a t/ha)	(25)a	(4.45a t/ha)	(23)a	(4.86a t/ha)
Leaf assessed:		L2		L2		L2	
Cultivar:		Triumph		Goldmarker		Koru	

(a)=see Table 2 *WAD= Weeks after drilling +WAT= Weeks after foliar spray
(b)=as % untreated

The mixture of flutriafol, ethirimol and thiabendazole, used as a seed treatment, gave high levels of mildew control in 3 trials in spring barley in 1985 resulting in significant increases in yield over untreated plots (Table 8). These increases were also significantly superior, in most instances, to those provided by the triadimenol-based formulation. When these seed treatments were followed by a foliar spray of a triazole fungicide, mildew control and yield were usually increased significantly over those obtained from the seed treatment alone. However, the highest level of control and yield increase were given by following the mixed group 2 and group 4 seed treatment (flutriafol + ethirimol) with foliar spray of the broad spectrum triazole, propiconazole.

Substituting a group 3 compound for a triazole as the first mildew treatment applied to winter barley in the spring serves to alternate modes of action if an autumn triazole-based seed treatment has been applied. Table 9 presents results from 3 trials which compared such programmes. The first programme evaluated the mixed group 2 and group 4 seed treatment followed by a foliar spray of a group 3 fungicide. The second programme used the same autumn seed treatment but followed this with a triazole-based (group 2) spring spray. The third programme used autumn triazole and spring triazole treatments for mildew control without alternating or mixing compounds with different modes of action.

Mildew control was best with the first programme where the alternating approach was followed. Nevertheless, the use of the mixture of flutriafol/ethirimol as the autumn seed treatment followed by a triazole spray in the second programme also provided better mildew control than the programme based on triazoles only. Mean yields from a total of nine trials

were similar for the first and second programmes, probably through the control of diseases other than mildew provided by flutriafol. Yields from the triazole only programme were lower although still greater than those from unsprayed plots.

TABLE 9

Control of mildew[a] and yield increase[b] in 1984 with different winter barley fungicide programmes.

Seed treatment	Foliar spray at GS31 WAT+	Trial 1 Lincs 6	Trial 2 Cambs 6	Trial 3 Cambs 4	% Yield*
Untreated	(actual % disease)	(5.2)a	(7.6)a	(30.3)a	(6.45 t/ha)
Flutriafol/ ethirimol/ thiabendazole	Fenpropidin + carbendazim	75c	86b	85d	113
Flutriafol/ ethirimol/ thiabendazole	Flutriafol/ carbendazim	69c	79b	65c	114
Triadimenol/ fuberidazole	Triadimefon/ carbendazim	42b	79b	43b	110
Leaf assessed:		L4	L2	L3	Igri
Cultivar:		Maris Otter	Maris Otter	Maris Otter	Sonja Maris Otter

+WAT = Weeks after foliar spray * = mean of 9 trials (a)=see Table 2
(b)=as % untreated

CONCLUSIONS

The use of mixtures of fungicides from different groups and alternating their use are accepted as a valuable means of delaying or preventing pathogen resistance. The ability of the agrochemical industry to develop mixtures depends on the availability of each component to a given company. Alternating the use of fungicides from different groups depends upon its promotion by the industry and compliance by the farmer.

Results from our trials demonstrate how a range of commercially available formulations from one or more chemical groups may be used alone, or in mixture, to control disease successfully despite the threat of fungicide resistance.

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FUNGICIDE SENSITIVITY CHANGES IN *RHYNCHOSPORIUM SECALIS* IN GLASSHOUSE EXPERIMENTS

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ABSTRACT

Carbendazim, prochloraz, propiconazole and triadimenol, applied as foliar sprays at 1.0 mg/l completely prevented infection on potted barley plants inoculated 36 h previously with conidia of *Rhynchosporium secalis* obtained from field plots after spraying twice with the respective fungicides in summer 1985. Conidia obtained from plants that received 0.1 mg/l (or less) in this test, and from lesions that developed on fungicide-treated plants in subsequent tests, were used to inoculate plants treated with a range of concentrations of the same fungicide. After four successive transfers, lesions of *R. secalis* developed on plants sprayed with 1000 mg/l carbendazim or triadimenol and 10 mg/l prochloraz or propiconazole. This decreased sensitivity to fungicide was still maintained after five further transfers through unsprayed plants. The adapted forms retained normal pathogenicity and ability to grow and sporulate *in vitro* and *in vivo*.

In agar plate tests, minimum inhibitory concentrations (MIC) from an initial isolate (from unsprayed field plots in 1985) were 0.3 mg/l for triadimenol and 0.01 mg/l for carbendazim, prochloraz and propiconazole. MIC values of isolates obtained after four passages through plants treated with the respective fungicides were >10 mg/l triadimenol, 0.3 mg/l carbendazim, 0.3 mg/l prochloraz, 0.3 mg/l propiconazole, indicating at least a thirty-fold shift in sensitivity *in vitro* to each fungicide.

The study was done to investigate the capacity for change in sensitivity. To date there is no evidence of any loss of disease control in the field.

INTRODUCTION

Leaf blotch, caused by *Rhynchosporium secalis*, is a common disease of barley, particularly in cool wet areas of the U.K., and can lead to serious reduction in yield when severe epidemics occur. Yield losses of 40% have been reported (James *et al*. 1968) but more commonly losses are less than 10%. Benzimidazole fungicides (benomyl, carbendazim, thiophanate-methyl) and sterol C-14 demethylation inhibitor (DMI) fungicides (prochloraz, propiconazole, triadimenol) are the most effective for control of *R. secalis* (Jordan *et al*. 1982; 1986) and are extensively used in the U.K., and elsewhere, for control of foliar and some stem-base diseases of barley. However, these fungicides are liable to produce shifts in the sensitivity of treated pathogens. Benzimidazole-resistant strains of *Pseudocercosporella herpotrichoides* and *Septoria tritici* are widespread in U.K. (King & Griffin 1985; Griffin & Fisher 1985), and shifts in triazole sensitivity have been reported for barley powdery mildew (*Erysiphe graminis*) (Butters *et al*. 1984).

Since no loss of disease control or shifts in sensitivity have been reported in R. secalis, it seems possible that use of either of these groups of fungicides alone on barley was too limited to exert sufficient selection pressure on the pathogen, or else the fungus is less adaptive, for no insensitivity has been reported in vivo or in vitro.

Hollomon (1984) tested 30 single-spore isolates of R. secalis and found the minimum inhibitory concentration of triadimenol ranged from 0.2 to 3.2 mg/l. All were controlled by triadimenol + fuberidazole applied as a seed treatment at manufacturers' recommended rate. As far as the authors are aware, no other reports of the response in vitro of R. secalis to DMI or benzimidazole fungicides have been published.

In order to investigate the capacity for change in sensitivity of R. secalis, glasshouse experiments were done to determine whether successive transfer of isolates of the pathogen through fungicide-treated plants could induce stable shifts in sensitivity. To date there has been no indication that these fungicides are not giving adequate control of leaf blotch in the field.

MATERIALS AND METHODS

Sources of inoculum from the field

The site, at Long Ashton Research Station (LARS), was used in 1983 - 84 for a winter barley experiment in which 3, 2 or 1 sprays of triazole fungicide had been applied to randomised plots across the area. In autumn 1984 the site was ploughed in such a way as to leave much stubble debris as a source of inoculum for a winter barley experiment in 1984-85. Leaves of winter barley cv. Igri, with symptoms of leaf blotch (Rhynchosporium secalis) were removed in June 1985 from five selected treatments in the field experiment that had received two applications (GS 31 + GS 39) of (i) carbendazim (0.25kg a.i. /ha), (ii) prochloraz (0.4kg a.i. /ha), (iii) propiconazole (0.125kg a.i. /ha), (iv) triadimenol (0.25kg a.i./ha) or (v) no fungicide sprays.

Glasshouse Transfer 1 (initial sensitivity of field inoculum)

Lesions of R. secalis from leaves in each field treatment were excised, floated on 20 ml glass distilled water at 11°C for 36h in the dark to provide conidial inoculum from the 5 sources listed above. The resultant spores in suspension were diluted to 50,000 spores /ml and used to inoculate, separately, batches of barley seedlings, cv. Igri, grown in pots (5 plants/pot) in a glasshouse,to the 5-leaf stage of development.

Following incubation for 36h at 15°C in a moist polyethylene tent, batches of eight replicate pots were sprayed with the same fungicide as that used in field plots. Plants were sprayed to maximum retention in a spray cabinet with fungicide concentrations of 10, 1, 0.1 or 0.01 mg a.i./l. Batches were also left unsprayed. Deposits were allowed to dry for 4 h, and the plants then returned to the polyethylene tent for a further 20 days, by which time symptoms of leaf blotch had developed on unsprayed plants. The percentage leaf area diseased was estimated visually on the two youngest leaves that were fully expanded at the time of spray application.

Glasshouse Transfer 2

Lesions were excised from fungicide-treated plants (0.1 and 0.01 mg/l doses grouped together for each fungicide) and from unsprayed plants, all

from glasshouse transfer 1, and sporulation induced as before. Conidia (150,000/ml) from the respective treatments were used to inoculate a fresh batch of barley plants cv. Igri, and the same procedure followed as for Transfer 1, except that fungicide concentrations were 100, 10, 1 and 0.1 mg/l.

Plants inoculated with conidia of R. secalis from untreated plants were again either left unsprayed or sprayed with 10 or 100 mg/l of each fungicide under evaluation.

Plants were incubated and disease assessed as before.

Glasshouse Transfer 3

Lesions were excised from plants treated with the highest fungicide concentration, and the same procedures followed. Plants were sprayed with 1000, 100, 10, 1 and 0.1 mg/l of the respective fungicides.

Glasshouse Transfer 4

This was a repeat of Transfer 3, using the same fungicide concentrations. In addition, to test the durability of the observed adaptations to fungicide treatment, conidia from lesions that developed on fungicide-treated plants were used to inoculate untreated plants; conidia were recovered from these, and further unsprayed plants were inoculated. This process was repeated at 3-weekly intervals throughout the experiment. Inoculum obtained from these plants was tested for fungicide response in comparison with inoculum from fungicide-treated plants as before. Descriptions of the inocula used are summarised in Table 1.

TABLE 1

Inoculum source for treatments used in Transfer 4

Inoculum	Source
A	Isolated from lesions of R. secalis taken from unsprayed plots (LARS field experiment), summer 1985, and stored as dried leaf specimens.
B	Isolated from lesions that developed on plants treated with the same fungicide in the previous glasshouse transfer 3.
C	Inoculum B, but transferred through unsprayed barley plants on two occasions prior to use.

For each fungicide the test series would be: A treated with 0, 10 and 100 mg/l; B and C treated with 0, 0.1, 1, 10, 100 and 1000 mg/l.

Fungicide sensitivity in vitro

Lesions were excised from diseased leaves after glasshouse Transfer 4, and from leaves that had not been exposed to fungicide in the field or laboratory, surface sterilised in sodium hypochlorite solution (1% available chlorine) + 0.01% vol/vol Tween 80, and placed on the surface of 0.6% distilled water agar (+ 100 mg/l streptomycin). Following incubation for 4 days at 17°C, the excised lesions were removed, the resultant discharged conidial masses transferred to 1 ml sterile water,

spread onto malt yeast agar amended with a range of fungicide concentrations (0.001 - 30 mg/l) and incubated for 7 days at 23°C. Germination and subsequent colony growth, from a sample of 100 conidia in each of four replicate agar plates were assessed microscopically and placed in one of three categories: (a) distinct colonies, (b) hyphal growth, (c) germ tubes stunted or lysed. Minimum inhibitory concentrations (MIC) were determined as the minimum dose to inhibit hyphal growth completely.

MIC values of some isolates were also obtained using the method described by Hollomon (1984), whereby single-spore isolates were obtained by streaking spore suspensions on malt yeast agar and transferring germlings to fungicide amended media after 7 days at 20°C.

RESULTS

Transfer 1

Symptoms of leaf blotch developed on all inoculated leaves of untreated plants and plants to which the four fungicides were applied at 0.1 or 0.01 mg/l, but not at higher concentrations (Table 2). The leaf areas diseased (%) on unsprayed plants averaged 19.6% (range 10 - 40%) whereas on the fungicide treated plants diseased areas did not exceed 15%.

Transfer 2

The mean diseased leaf area on untreated plants in this test was 18.9%. Symptoms of leaf blotch developed on all plants sprayed with fungicide at 0.1 mg/l, although diseased areas were less (range 1.2 - 13.1%). A few lesions developed on some plants sprayed with 1 mg/l propiconazole or 10 mg/l of triadimenol and carbendazim (Table 2).

TABLE 2

Effects of previous exposure to a fungicide on the sensitivity of *R. secalis* to foliar sprays of that fungicide

Test No.	Inoculum pre-treatment			Highest spray conc.(mg/l) allowing any disease on sprayed seedlings			
	Field sprays	Seedling transfers sprayed	Seedling transfers unsprayed	carben-dazim	triad-imenol	propic-onazole	proch-loraz
1	*0	0	0	0.1	0.1	0.01	0.01
	2	0	0	0.1	0.1	0.01	0.01
2	2	1	0	10	10	1.0	0.1
3	2	2	0	100	1000	10	10
4	≠2	3	0	100	1000	10	10
	ϕ2	3	2	100	1000	10	10
	ϕ2	3	5	100	1000	10	10

* Initial inoculum from untreated field plots in 1985 (Inoculum A); ≠ Inoculum B; ϕ Inoculum C; (see Table 1).

No disease symptoms developed on plants that received inoculum from untreated plants (Inoculum A) and sprayed at 10 and 100 mg/l.

Transfer 3

The mean diseased leaf area on untreated plants in this test was 16.3%. Prochloraz or propiconazole applied at 10 mg/l and carbendazim or triadimenol applied at 100 mg/l gave effective disease control, whereas all fungicides applied at 1 mg/l failed to reduce disease severity.

Transfer 4

Response to fungicides was similar to that observed in Transfer 3. There were no differences in disease response between inoculum from previously fungicide-treated plants (Inoculum B) and inoculum subsequently passaged through untreated plants (Inoculum C). Symptoms did not develop on plants inoculated with Inoculum A when sprayed with 10 or 100 mg/l fungicide (Tables 2,3).

TABLE 3

Disease* on fungicide-sprayed barley plants inoculated with isolates of *R. secalis* previously subjected to different degrees of fungicide exposure

Fungicide	Inoculum source†	Rate of fungicide application (mg/l)				
		0	1	10	100	1000
Carbendazim	A	11	–	0	0	–
	B	7 ns	10 ns	5	2	1
	C	9 ns	5	2	3	0
Triadimenol	A	15	–	0	0	–
	B	16 ns	11 ns	5	10	1
	C	11 ns	7	7	6	1
Prochloraz	A	13	–	0	0	–
	B	16 ns	6	2	0	0
	C	9 ns	3	2	0	0
Propiconazole	A	18	–	0	0	–
	B	24 ns	7	4	0	0
	C	11	2	2	0	0

* Values are % diseased leaf area (mean of 30 leaves); – not tested;
† See Table 1.
All values except those marked ns are significantly different (P=0.05) from unsprayed (A/0) within each fungicide treatment.

Fungicide sensitivity in vitro

Growth from the initial sensitive isolate from unsprayed field plots (A) was completely inhibited on malt yeast agar amended with 0.3 mg/l triadimenol, whereas all isolates obtained from lesions on triadimenol-treated leaves following the fourth transfer, irrespective of concentration applied or subsequent passaging through untreated barley leaves, produced distinct colonies or hyphal growth on agar containing 10 mg/l triadimenol. Sparse hyphal growth at 30 mg/l triadimenol was observed from some conidia in the population sampled from two isolates (B/100 and C/0, Table 4). Using the method of Hollomon (1984) these isolates produced visible colonies at 12.8 mg/l triadimenol, the highest concentration tested (D.W. Hollomon, pers. comm.).

TABLE 4

Growth/ of R. secalis on malt yeast agar amended with triadimenol

Inoculum/ triadimenol concentration (mg/l)ϕ	Concentration of triadimenol in agar (mg/l)				
	0	1	3	10	30
A/0*	100	0	0	0	0
B/0	100	100	90	75	0
B/1	100	100	80	80	0
B/10	100	100	95	35	0
B/100	100	100	100	50	20
B/1000	100	100	95	100	0
C/0	100	100	100	100	2
C/10	100	100	85	50	0
C/100	100	100	85	50	0

/ Values are % of conidia forming colonies or showing hyphal growth.
ϕ Triadimenol concentrations (mg/l) applied to plants from which isolates were obtained.
* See Table 1, B and C obtained from plants previously treated with < 100 mg/l triadimenol.

Growth of the initial isolate (A) was completely inhibited on agar amended with 0.01 mg/l carbendazim, propiconazole or prochloraz (Table 5), whereas isolates obtained from lesions on fungicide-treated plants had MIC values of 0.3 mg/l carbendazim, 0.3 mg/l prochloraz and 0.3 mg/l propiconazole (Table 5).

TABLE 5

Growth/ on fungicide-amended agar of isolates of R. secalis with and without previous exposure to the fungicide

Fungicide	Inoculum source*	Concentration (mg/l)of fungicide in agar									MIC value (mg/l)
		0.001	0.003	0.01	0.03	0.1	0.3	1	3	10	
Triadimenol	A/0	100	100	100	100	85	0	0	0	0	0.3
	B/100	100	100	100	100	100	100	100	100	70	>10.0
Carbendazim	A/0	100	85	0	0	0	0	0	0	0	0.01
	B/100	100	100	100	85	65	0	0	0	0	0.3
Prochloraz	A/0	95	40	0	0	0	0	0	0	0	0.01
	B/10	100	100	100	70	25	0	0	0	0	0.3
Propiconazole	A/0	90	60	0	0	0	0	0	0	0	0.01
	B/10	100	100	90	85	15	0	0	0	0	0.3

* A and B defined in Table 4; 0, 10, 100 indicate fungicide spray concentrations applied at fourth transfer.
/ Values are % of conidia forming colonies or showing hyphal growth.

DISCUSSION

Throughout the glasshouse tests complete control of isolates of Rhynchosporium secalis obtained from glasshouse grown plants that had not received fungicide applications was achieved with sprays containing 10 mg/l of each test fungicide. The in vivo assays did, however, show the progressive ability of R. secalis to produce lesions on fungicide-treated plants when the inoculum had been derived from leaves previously treated with that particular fungicide. After three successive transfers, lesions were produced on plants treated with 100 mg/l carbendazim and 1000 mg/l triadimenol. Loss of sensitivity (expressed as in vivo MIC ratios), was not shown to the same extent with prochloraz or propiconazole but, by the fourth transfer, lesions developed on plants sprayed with 10 mg/l of these fungicides whereas initially complete control was given by 0.1 mg/l. Transfer of such selected isolates through untreated plants (on 5 occasions by the time this report was prepared) did not restore the original sensitivity, indicating a stability of response in the absence of fungicide selection pressure.

In vitro studies on malt yeast agar, using conidia of R. secalis produced from lesions obtained initially, and after the fourth transfer through fungicide-treated plants, showed a 30-fold decrease in sensitivity to triadimenol, carbendazim, prochloraz and propiconazole. Hollomon (1984) demonstrated a variation in sensitivity (MIC in vitro) of single-spore field isolates of R. secalis to triadimenol within the range 0.2 - 3.2 mg/l. The reduction in sensitivity to triadimenol observed in vitro in our assays (from 0.3 to > 10 mg/l) is probably sufficient to explain the reduced fungicide efficacy observed in the in vivo tests. There are no base-line fungitoxicity data for carbendazim, prochloraz and propiconazole, and although a similar relative shift in MIC values from 0.01 to 0.3 mg/l occurred, it could be argued that both values fall within the sensitivity range shown by the "wild-type" population. Nevertheless, fungicide efficacy did decrease in vivo. To relate these experimental glasshouse findings to field situations, monitoring of fungicide sensitivity of R. secalis populations in field crops is required and work along these lines is currently in progress at Long Ashton.

Loss of effectiveness of fungicides against R. secalis in the field has not been reported and the practical implications of these results are difficult to determine. In barley crops these fungicides are seldom, if ever, applied repeatedly in one season, and certainly not at the low concentrations used initially in these tests. Commonly, however, two or more of these fungicides are used per season, often as mixtures or co-formulations which include fungicides from other groups. Future studies will determine to what extent cross-sensitivity between the DMI's occurs and how this may be influenced by fungicides from other groups.

It is extremely unlikely that field populations of R. secalis would, within one season, be subjected to the selection pressure provided by our sequential fungicide test regimes. However, if isolates subjected to moderate selection pressure over one season develop a low, but stable, level of decreased sensitivity, and if such strains can overwinter, there is the possibility that further selection pressure in subsequent years may further reduce the sensitivity of this pathogen to fungicides.

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INVESTIGATIONS ON SENSITIVITY- AND VIRULENCE-DYNAMICS OF ERYSIPHE GRAMINIS F. SP. TRITICI WITH AND WITHOUT TRIADIMENOL TREATMENT

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ABSTRACT

The triadimefon-sensitivity of a powdery mildew population in a winter wheat field varied during the course of the season even in the absence of fungicide pressure. The mildew population of a triadimenol-treated plot also changed continuously during the season but fungicide pressure did not induce any clearly visible shift from that in an unsprayed plot. Under the influence of triadimenol the most sensitive isolates were only of low frequency in the population and isolates with low sensitivity were found that could not be detected in the control plot.
At the beginning of the trial the powdery mildew population consisted of a race mixture with a pronounced focus on combined virulence against Pm 8, Pm 5, Mli and general resistance. The virulence diversity decreased with the course of time. Triadimenol seemed to have no influence on the virulence selection.
In general sensitivity values and virulence-type seemed not to be correlated.

INTRODUCTION

Much research has been done, and many reports have been published, on the topic of triazole fungicides. Wolfe (1985) and De Waard et. al. (1986) found that intensive triazole use leads to the risk of resistant powdery mildew populations developing and hence to decreased field performance of a fungicide. Within the last four years much effort has been devoted to testing numerous mildew samples from fields where the farmers claimed unsatisfactory performance. As we know today many factors influence the effectiveness of a fungicide, and poor efficacy of a spray in the field is not necessarily correlated with fungal resistance.

We thought it interesting, therefore, to study more intensively the dynamics within the powdery mildew population of a clearly defined area. The purpose of our research was to observe any changes within a mildew population (Erysiphe graminis DC f. sp. tritici) as a result of heavy fungicide pressure during the course of a season (1985).

MATERIALS AND METHODS

Field trials

Two one hectare areas, for a control plot and a triadimenol-treated plot, were located within a field of winter wheat (cv. Kanzler) of several hectares. The treated plot was sprayed with '(R)Bayfidan' (250 EC, 0,5 l/ha) whenever 5 % new infection had established itself (4 treatments during the season). During the whole test period, information and data concerning climatic conditions (temperature, rainfall, rel. humidity), nutrition (N-fertilizer) and plant growth were recorded.

Collection of single pustules

Single pustules were collected from the plots (100 pustules per plot), on six dates during the season (see Table 2). A young sporulating pustule was cut out of a leaf and put into a glass vial containing six detached leaves of cv.Kanzler. The spores of the pustule were dusted onto the fresh leaves by shaking the vial several times within one day. Afterwards the leaves were transfered to Petridishes containing water agar. After one week of incubation most of the samples had produced new powdery mildew spores. The six leaves per pustule sample, bearing new spores, were used as inoculum in the following sensitivity and virulence tests. No further subcultures were made.

Sensitivity test

Wheat plants (cv. Kanzler), were cultivated for one week under standardized conditions (20° C, 80 % rel. humidity). Ten detached primary leaves were arranged in plastic boxes (7 x 4,5 cm). Six boxes were needed for each sample - 1 control, 5 for a concentration range of triadimefon, '(R) Bayleton' 125 EC. The boxes with the leaves were sprayed under standard conditions with blank formulation or various triadimefon concentrations. After the leaves had dried the six boxes per sample were inoculated under a settling tower. The boxes were incubated for one week under standard conditions (18° C, 80 % rel. humidity).

After this time disease assessments were made my estimating the percentage of leaf area covered with powdery mildew. The data were transformed to probits and plotted against the logarithms of the doses in a weighted probit analysis. The ED 50 and ED 95 values obtained were used to characterise each sample. All data are presented as a frequency distribution of the ED values for single pustule isolates (see Fig. 1 and 2).

Virulence test

Detached primary leaves of wheat cultivars possessing different known resistance genes (see Table 1) were arranged in a circle in a round plastic box (22 cm Ø) containing water agar. The box was inoculated under a settling tower. After one week of incubation under standard conditions (18° C, 80 % rel. humidity) the percentage of leaf area covered with powdery mildew was estimated and the type of infection noted. Table 2 shows the frequency distributions of the virulence genes within samples of the mildew population taken on different dates in the season.

RESULTS AND DISCUSSIONS

Data from both plots taken before the test started clearly demonstrated that there was an evenly distributed population of mildew sensitivities. However, the triadimefon sensitivity of the population changed during the course of the season (see Fig. 1 and 2).

The triadimefon sensitivity of the powdery mildew population did not change between the first and the second sampling dates but data from the third sampling indicate that the whole mildew population was "on the move" with a trend towards lower sensitivities whether sprayed or not. In the middle of June (June 18) the distribution of the ED values from the control plot had a narrow concentration range. This date represents the lowest level of sensitivity found, because, on average, the ED values of the later isolates (especially July 1) were lower again. These results were in parallel with the observation that mildew in the control plot rapidly increased, and from July 1 on practically no green leaf area was available. The mildew, under deteriorating conditions, then began to produce cleistothecia.

Concerning the triadimenol influence on the powdery mildew population during a season the following facts should be emphasized:

Neither a disruptive, nor a clearly-pronounced directional, shift could be detected in the mildew population of the triadimenol plot compared to that of the control.

A comparison of the frequency distributions of the sensitivity data from the control and the triadimenol treated plots shows that in the treated plot the frequency of isolates with lower sensitivity increased and those with higher sensitivity decreased from the date of the first treatment. In the control plot the sensitivity values had an almost normal distribution, whereas in the treated plot the most sensitive part is missing and the distribution curves have a "cut-off" appearance (esp. June 18, see Fig. 1). To a large extent the sensitivity of the populations in both plots had equal concentration ranges but showed different frequency peaks. Only a few isolates with sensitivites lower than any present in the control plot were found in the treated plot.

Comparing the frequency distributions of the ED 50 values over the season one can see that the proportion of sensitive isolates increased again in both plots between the June 18 and July 1 (see Fig. 2). In contrast to the control, the basic population of the treated plot did not shift, as a whole, towards greater sensitivity since the ED 50 concentration ranges with the greatest frequency were identical for both sampling dates. However, an increased number of sensitive isolates were found on the July 1, compared with June 18.

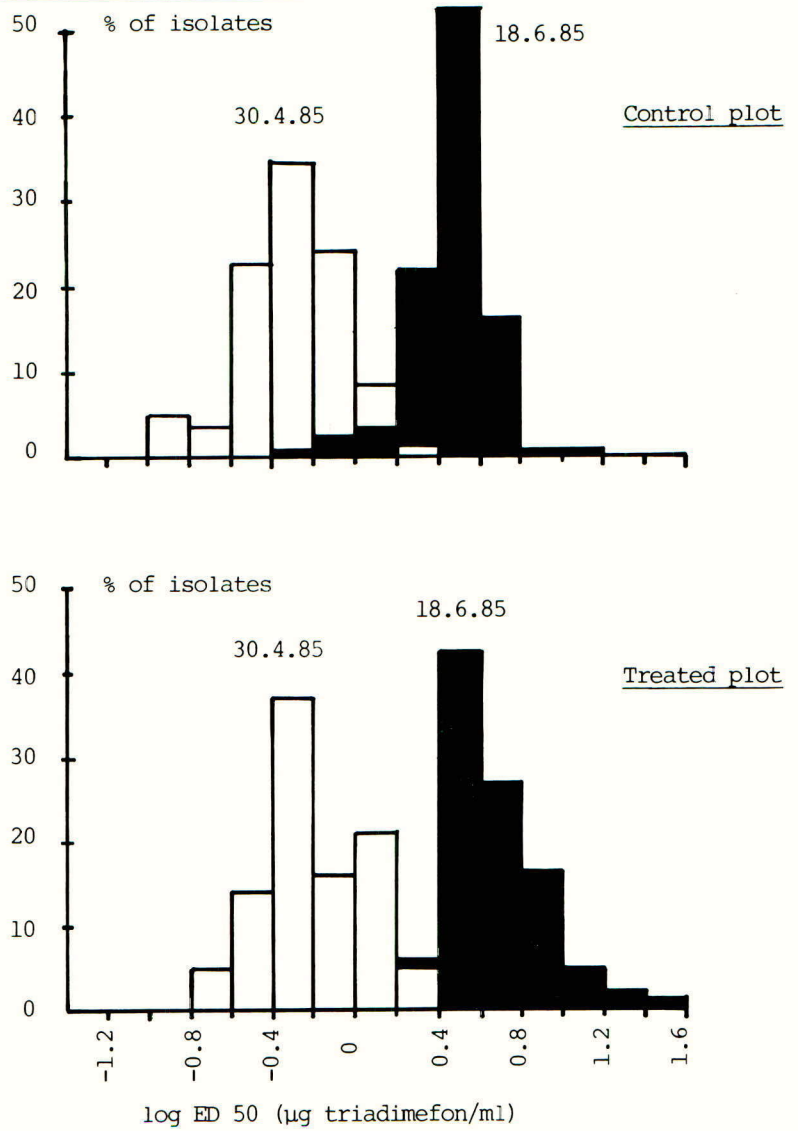
One should bear in mind that towards the end of the season the plants in the control plot were in a different physiological condition compared with those in the triadimenol-treated plot. In the treated plot the powdery mildew continuously found strong, green plants and sufficient leaf area for propagation because of a low population density. The mildew of this plot was certainly better nourished and more vigorous than mildew in the untreated plot that was forced to undergo sexual reproduction due to a severe lack of space and nutrients.

In our opinion an overlap of two factors occurred - sensitivity and vitality. An exact analysis of the variation of these parameters is not possible from the data available. One should realize the possibility that two distinct forces were operating whose effects may have the same result, namely a lower sensitivity towards triazole fungicides.

When the test started the powdery mildew population consistend of a mixture of races having a marked preponderance of combined virulence against the Pm 5, Pm 8, Mli and general resistance factors. The latter two factors alone lead to moderate intrinsic resistance (Table 2). It is also known that the genes Pm 5 and Pm 8 are no longer effective and that the frequency of Mli virulence is very high in the mildew population (Zimmermann et al., 1984). Therefore nearly all isolates from the field population attacked the cultivars Disponent, Diplomat, Janus and Kormoran. The variety Kanzler, without any known resistance gene, was also generally attacked vigorously. The tests revealed that a remarkable proportion of isolates of the population were capable of attacking Maris Huntsman, probably because this variety has been grown in the trial region.

A broad variation of different virulences was present at the first, and especially at the second, sampling date. As the stand of plants was not yet closed at these dates, one can assume that the powdery mildew present was typical for the whole area and not specific to that field. On the May 13, some

Fig.1 Frequency distributions of ED 50 values for triadimefon amongst single pustule isolates sampled on two dates



Field data

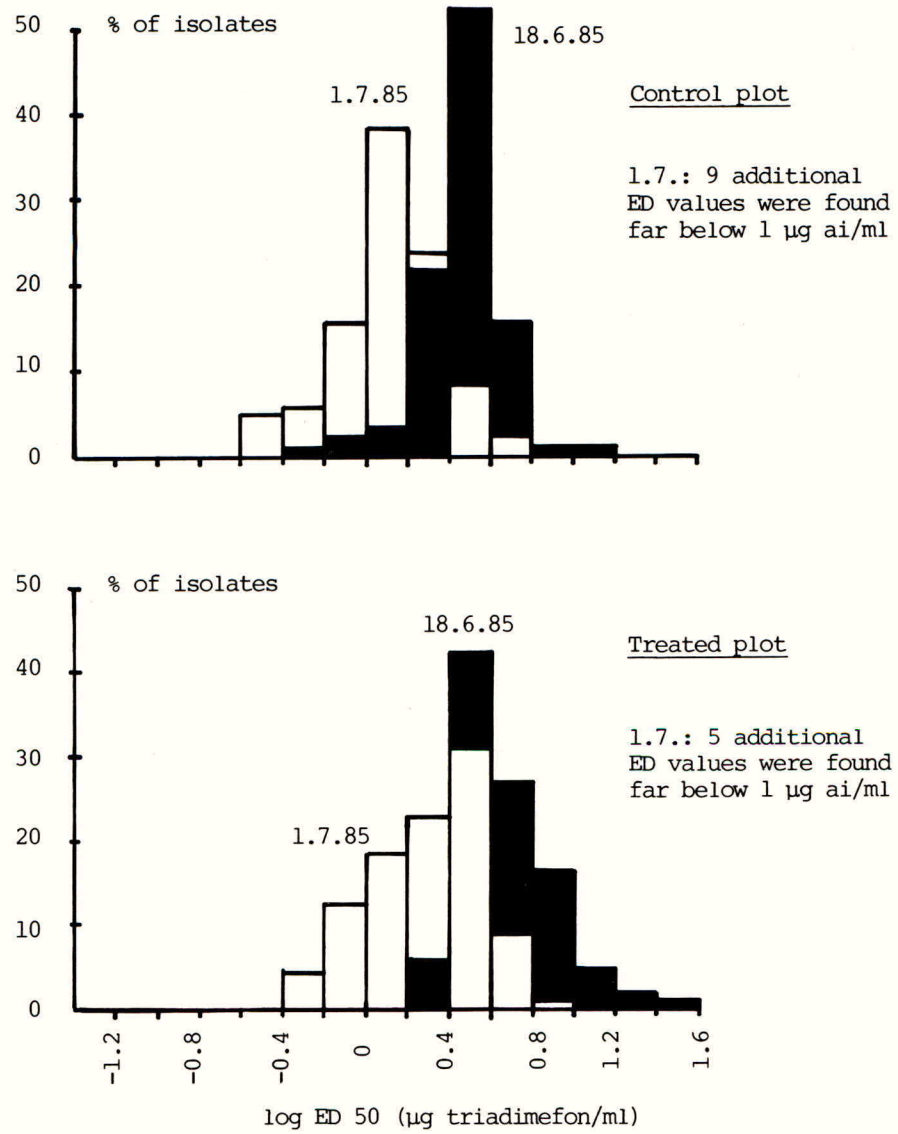
triadimenol sprays: 21.5. and 3.6.85

sampling and assessment: 18.6.85

% mildew in control plot: 6.7

% effectiveness on leaves (Abbott) in treated plot : 72

Fig. 2 Frequency distributions of ED 50 values for triadimefon amongst single pustule isolates sampled on two dates



Field data

triadimenol sprays: 21.5.
3.6.
19.6.

sampling and assessment: 18.6.
1.7.

plot	18.6.	1.7.85
% mildew in control plot	6.7	30
% effectiveness (Abbott) in treated plot	72	90
	on leaf	on whole plant

isolates were found that grew on all test cultivars.

Whether triadimenol treatment was applied or not this diversity of virulences rapidly decreased from the third sampling date. In both plots the virulence factors selected for were those against Pm 5, Pm 8, Mli and general resistance.

From a review of all the virulence distributions during the course of the trial the conclusion can be drawn that the fungicide triadimenol apparently did not favour the selection of particular virulence factors.

The question follows as to whether there is any direct connection between triadimefon sensitivity and virulence. If the virulence patterns of sensitive isolates are compared with those of lower sensitivity, with or without respect to the plot they originated from, it is found that there were no differences in their ability to attack the range of differential wheat cultivars.

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This paper is an extract of a report that will soon be published in full length in Pflanzenschutz Nachrichten Bayer 1986.

Table 1: Differential spectrum of wheat cultivars used for the virulence test

cultivar	Powdery mildew resistence gene(s)
Winter wheat	
Kanzler	none
Disponent	Pm 8
Diplomat	general resistence
Maris Huntsman	Pm 2+6
Kormoran	Mli
Spring wheat	
Walter	Pm 2+4b+6
Turbo	Pm 1+2+4b ?*
Janus	Pm 5 ?
Mephisto	Pm 1+2

*? not yet completely cleared up

Table 2: Distribution of virulence types[(a)] within individual samples of mildew pustules taken during the course of the season

SAMPLING DATE	PLOT	DIFFERENTIAL VARIETIES	VIRULENCE PATTERN [(b)]																											
		Kanzler	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		Disponent	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+		+	+
		Diplomat	+	+	+	+	+	+	+	+		+	+	+	+		+	+	+	+	+	+	+	+	+	+	+		+	+
		M.Huntsman				+		+					+	+		+		+		+	+	+	+	+	+		+	+		
		Kormoran	+		+	+	+	+	+	+	+				+	+	+	+		+	+	+	+		+		+		+	+
		Walter							+				+				+	+	+	+	+				+			+	+	+
		Turbo			+					+		+	+			+	+	+			+	+	+					+		+
		Janus	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+			+	+	+
		Mephisto					+	+		+			+	+					+		+	+			+				+	+
30.4.85	Control		46	10	9	6	5	4	3	2	2	1	1	1	-	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-
	Triadimenol		60	7	2	10	3	3	2	-	-	-	-	1	1	-	3	-	-	2	1	1	1	-	-	-	-	-	-	-
13.5.85	Control		18	1	7	12	-	7	3	-	-	2	-	-	-	-	4	3	-	1	8	4	2	1	3	1	-	-	-	-
	Triadimenol		16	2	5	14	5	7	1	3	-	-	-	-	1	-	2	1	-	-	3	7	2	1	3	-	-	-	-	-
3.6.85	Control		25	-	3	6	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-
	Triadimenol		43	2	2	4	1	5	-	-	-	-	-	2	-	-	2	-	-	-	-	-	1	-	-	-	1	1	-	-
18.6.85	Control		48	-	4	11	-	3	-	-	-	-	-	-	-	-	1	-	-	-	1	2	-	-	-	-	-	-	-	-
	Triadimenol		65	1	3	12	3	3	1	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	1	-	1	-	1	-
1.7.85	Control		46	-	4	13	3	18	2	-	-	-	-	1	-	-	-	-	-	-	-	-	3	1	1	-	-	-	1	-
	Triadimenol		61	2	5	10	6	4	-	-	1	-	-	-	-	-	1	1	-	-	1	1	-	-	1	-	-	-	-	2
16.7.85	Control		10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Triadimenol		43	-	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(a) Figures are the number of single pustules from one plot showing a particular virulence pattern
(b) "+" = isolate grows on variety

CROSS-RESISTANCE OF TRIADIMENOL-RESISTANT FUNGAL ISOLATES TO OTHER STEROL C-14 DEMETHYLATION INHIBITOR FUNGICIDES

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ABSTRACT

Triadimenol resistant isolates of Cladosporium cucumerinum, Sphaerotheca fuliginea and Pyrenophora teres were examined for their sensitivity to twelve other fungicides considered to have a similar mode of action. The isolates of C. cucumerinum and S. fuliginea showed cross-resistance to all sterol demethylation inhibitors (DMIs) but the degree of cross-resistance to individual fungicides differed greatly. The resistant isolate of P. teres showed a more restricted range of cross-resistance than resistant isolates of the other two pathogens. None of the resistant isolates showed cross-resistance to chloraniformethan, a compound having some structural similarity to triforine, a known DMI. Relationships between degrees of cross-resistance and chemical structure are discussed in terms of their implication for the modes of action, mechanisms of resistance and practical use of these fungicides.

INTRODUCTION

The sterol demethylation inhibitor class of fungicides (DMIs) are a group of structurally diverse compounds which appear to have a common mode of action-inhibition of the C-14 demethylation stage in the biosynthetic pathway from lanosterol to ergosterol (Langcake et al. 1983; Kato 1986). They have great importance in the chemical control of many crop diseases because of their high level of efficacy in the field, often coupled with systemic properties and a broad spectrum of activity. Since the introduction of triforine in 1971 the number of DMI fungicides available has increased considerably and others are currently under development (Kuck & Scheinpflug 1986).

Early laboratory investigations into the likelihood that resistance to these fungicides might develop (Fuchs & de Waard 1982; de Waard & Fuchs 1982) indicated that DMI-resistant mutants were less pathogenic than the sensitive forms, leading to the view (Fuchs & Drandarevski 1976) that resistance to this group of fungicides was unlikely to become a problem. More recently, however, forms with decreased sensitivity have arisen in crops, and in some instances, this has resulted in a reduction in the efficacy of some of these fungicides against pathogens such as barley powdery mildew (Butters et al. 1984). Resistance to fenarimol in cucumber powdery mildew has already proven to be of practical importance (Schepers 1983; Huggenberger et al. 1984). Resistance to triadimenol in net-blotch has been reported in New Zealand (Sheridan et al. 1985); however, in Europe triadimenol has never provided good control of this disease.

As a rule amongst agricultural fungicides mutants selected for resistance to one fungicide, either in the laboratory or field, frequently show reduced sensitivity to other compounds with the same mode of action, i.e. they are cross-resistant to those compounds. Studies on

cross-resistance within DMI fungicides, reviewed by de Waard & Fuchs (1982), have shown that cross-resistance within this group is usually, but not invariably, present. Work using laboratory-induced mutants of Ustilago maydis (Barug & Kerkenaar 1984) similarly showed that although cross-resistance between DMIs is the norm, exceptions occur and cross-resistance between two fungicides was not always reciprocal.

In the present study triadimenol resistant isolates of Cladosporium cucumerinum (gummosis of cucurbits), Sphaerotheca fuliginea (cucumber powdery mildew) and Pyrenophora teres (net blotch of barley) were examined for their cross-resistance towards twelve other DMI fungicides. The isolates of S. fuliginea and P. teres, both important pathogens, have recently been obtained from the field. The isolate of C. cucumerinum comes from a culture collection and its low sensitivity to triadimenol was observed in laboratory assays. Chloraniformethan, a fungicide whose mode action is unknown but with a molecular structure similar to that of triforine (see Fig. 1), was also included.

This work is part of a larger programme of research at Long Ashton aimed at understanding the mechanisms of selectivity and resistance to DMI fungicides.

MATERIALS AND METHODS

Fungal isolates

Cladosporium cucumerinum

Isolate 840905, triadimenol sensitive, was kindly supplied by Dr A. Fuchs, Phytopathology Laboratory, University of Wageningen. Isolate 49628, triadimenol resistant, was a spontaneous mutant which originated in the culture collection of the Commonwealth Mycological Institute and was provided by Dr Louise Cooke, Department of Agriculture for Northern Ireland, Belfast. Single spore isolates, made from the original cultures, were used in these studies.

Pyrenophora teres

Isolates 21/84 7, triadimenol sensitive, and 37/84 1, triadimenol resistant, were obtained from Dr J.E. Sheridan, Botany Department, Victoria University of Wellington, New Zealand. They were derived from samples of infected barley seed. The original isolates, particularly that of the resistant form, varied from time to time in the morphology, pathogenicity, and symptoms produced on host plants. The cultures used in these studies were isolated from single typical 'netted' lesions on inoculated barley seedlings; however, some morphological variation in vitro was still encountered.

Sphaerotheca fuliginea

Isolate ENG 01, triadimenol sensitive, was supplied by Mrs M.A. Collins, Lilley Research Centre, Windlesham, and originated from the Jealott's Hill Research Station of ICI, where it was used for fungicide screening. Isolate H3, triadimenol-resistant, was made by the author during a survey carried out in 1985, jointly with Dr D.M. Derbyshire, ADAS, Cheshunt, to monitor the sensitivity to imazalil of populations of S. fuliginea in commercial glasshouses within the Lea Valley area.

Maintenance of cultures

During these investigations isolates of C. cucumerinum and P. teres were maintained by regular transfers on agar media. Cultures of S. fuliginea were kept on cucumber plants grown in isolation.

Fungicides

The fungicides included in this study are listed in Table 1. Unformulated, technical grade samples, supplied by the manufacturers, were used except for DPX-H6573 (proposed common name flusilazol) for which only a 40% a.i. emulsifiable concentrate formulation was available.

Measurement of fungicide sensitivity

Sphaerotheca fuliginea

Cucumber plants (cv. Suttons Improved Telegraph) were grown to the three leaf stage in a mildew free glasshouse. Individual leaves were removed and inoculated with mildew conidia by gently stroking their adaxial surface with a cucumber leaf heavily infected with the appropriate isolate. Discs (1 cm diam.) were cut from the inoculated leaf and batches of four floated on 10 ml of an aqueous solution of a fungicide contained in a 6 cm diam. petri dish. Inoculated discs were floated on distilled water as controls. A single inoculated leaf provided sufficient discs to test five fungicides; triadimenol was included in all tests as a standard. In an initial test all fungicides were used at 10, 1, 0.1 and 0.01 mg/l. Results from this initial test were used to identify what concentrations were needed in a two-fold dilution series to provide satisfactory estimates of EC50 values.

The discs were incubated (for 5-6 days) in a growth cabinet at 23°C with a 16h daylength under fluorescent light. Disease development was assessed as the percentage area of each disc visibly infected with mildew. Untreated discs consistently showed c. 90% of their area diseased. Mean disease levels were calculated for each treatment and expressed as a percentage of that on controls. Percentages were plotted against fungicide dose on a log-probability scale and EC50 values estimated from dosage/response curves fitted by eye.

Cladosporium cucumerinum

Glucose-yeast agar plates were surface seeded with conidia and incubated overnight to give a felt of non-sporing mycelium. Two plugs (5 mm diam.) cut from those plates were placed on opposite sides of each of two duplicate plates of glucose-yeast agar containing fungicide, added from a stock solution in acetone after autoclaving. Control plates without fungicide were inoculated in the same way. Initially a ten-fold dilution series (100, 10, 1, and 0.1 mg/l) was used followed, in a second test, by a two-fold dilution series designed to encompass the estimated EC50 concentration. The plates were incubated at 23°C and colony size measured after five and thirteen days. The mean values for increase in size of the four colonies per treatment over this eight day period were expressed as a percent of that obtained on control plates. EC50 values were determined graphically as described for S. fuliginea.

Pyrenophora teres

Two mycelial plugs (5 mm diam.), cut from the periphery of a young colony growing on glucose-yeast agar, were placed on opposite sides of a plate of malt extract agar with or without a fungicide at concentrations of 50,5,0.5, and 0.05 mg/l. Duplicate plates were used for each fungicide concentration. Colony growth between the third and fifth day of incubation (23°C) was measured and the data analysed to give EC50 values as described above for C. cucumerinum. Only a ten-fold dilution series of fungicide concentrations was used in these initial experiments and consequently the EC50 values are less precise than those obtained for C. cucumerinum.

TABLE 1

Sensitivity of fungal isolates to thirteen DMI fungicides and chloraniformethan

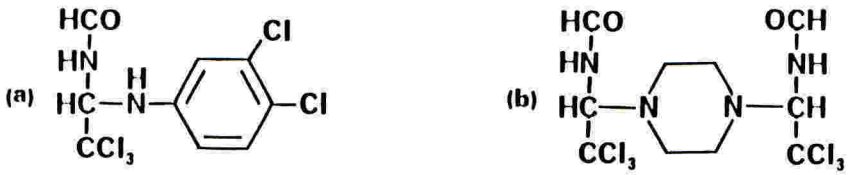
Fungicides	Cladosporium cucumerinum			Sphaerotheca fuliginea			Pyrenophora teres		
	EC50[a]		RF[c]	EC50		RF	EC50		RF
	S[b]	R[b]		S	R		S	R	
Triadimenol	0.3	85.0	283	0.01	0.1	10	2.0	25.0	12.0
Triadimefon	0.4	>100.0	>250	0.007	0.7	100	26.0	16.0	0.6
Diclobutrazol	0.4	>100.0	>250	0.008	0.3	38	3.0	0.5	0.2
Penconazole	0.02	58.0	2900	0.002	0.3	150	0.07	0.2	3.0
Propiconazole	0.02	4.0	200	0.03	0.4	13	0.8	0.4	0.5
Flutriafol	0.1	>100.0	>1000	0.0004	0.3	750	2.0	6.0	3.0
DPX-H6573	0.04	5.0	125	0.03	0.5	17	0.06	0.02	0.3
Fenarimol	0.2	>100.0	>500	0.002	0.08	40	0.01	2.0	200.0
Nuarimol	0.2	>100.0	>500	0.002	0.09	45	0.05	3.0	60.0
Imazalil	0.04	4.0	100	0.02	0.2	10	0.1	0.03	0.3
Prochloraz	0.01	2.0	200	0.09	0.3	3	0.004	0.004	1.0
Buthiobate	0.3	>100.0	>333	0.1	0.7	7	0.8	25.0	31.0
Triforine	3.5	>100.0	>29	0.09	0.8	9	38.0	>50.0	>1.3
Chloraniformethan	20.0	36.0	1.8	0.4	0.6	1.5	6.0	8.0	1.3

[a] EC50 - concentration mg/l required to reduce fungal development by 50%.

[b] One isolate sensitive (S), the other resistant (R) to triadimenol.

[c] RF - resistance factor = EC50 for resistant isolate/EC50 for sensitive isolate.

Fig. 1. Structure of chloraniformethan (a) and triforine (b).



RESULTS

The degree of fungicide resistance shown by the triadimenol-resistant isolates was expressed by calculating a resistance factor (RF) for each fungicide. This was the ratio between the EC50 values of the triadimenol-resistant isolate and the corresponding triadimenol-sensitive one. RF values, together with the EC50 values from which they were derived, are listed in Table 1. The data have not yet been fully analysed and so S.E.s for individual EC50 values are not given but preliminary indications are that S.E.s for S. fuliginea and C. cucumerinum are c. 15% of the EC50 value and those for P. teres c. 25%.

The degrees of resistance to triadimenol differed considerably with RF values of 283, 10 and 12 for C. cucumerinum, S. fuliginea and P. teres respectively. Although precise RF values could not always be obtained with C. cucumerinum, on account of the limited water solubility of some fungicides and the low sensitivity of the triadimenol-resistant isolate of this pathogen, some cross-resistance occurred to all DMI fungicides. However, compared with triadimenol RF values were significantly ($P = 0.05$) lower for DPX-H6573, imazalil, prochloraz and propiconazole, and higher for penconazole and flutriafol. Different degrees of cross-resistance were observed in S. fuliginea to all DMI fungicides, but as with C. cucumerinum, prochloraz showed a significantly lower level; again the RF values for penconazole and flutriafol were markedly higher. The pattern of cross-resistance shown by P. teres was quite different. Obvious cross-resistance occurred only towards three other DMI fungicides, fenarimol, nuarimol and buthiobate. In fact, negatively correlated cross-resistance ($RF < 1$) was observed for many fungicides.

Resistance factors are ratios and independent of absolute levels of sensitivity. The resistant isolate of S. fuliginea was 250 times more sensitive to triadimenol than the resistant isolate of P. teres yet both showed similar RF values for this fungicide. Similarly P. teres was 30 times more sensitive to penconazole than to flutriafol yet showed a similar RF value for both. The higher RF values obtained with the triadimenol-resistant isolates for certain other fungicides arises not so much from their lower sensitivity to these compounds, compared with that for triadimenol, but from the much greater sensitivity of the corresponding triadimenol-sensitive isolate. This is particularly true of C. cucumerinum for penconazole and S. fuliginea for flutriafol.

The triadimenol-sensitive isolates varied widely in the degree of sensitivity to the other test fungicides, EC50 values ranging from 0.4-0.0004 mg/l for S. fuliginea, 20-0.01 mg/l for C. cucumerinum and

38.0-0.004 mg/l for P. teres. Against the majority of the fungicides, S. fuliginea proved the most sensitive and P. teres the least sensitive. However, differences in the order of sensitivity of species occurred with fenarimol, nuarimol, prochloraz, propiconazole and DPX-H6573. Individual fungicides differed in their spectrum of activity. For example, prochloraz, the most active compound against both C. cucumerinum and P. teres, was not very effective against S. fuliginea whilst flutriafol, by far the most effective against S. fuliginea, was not particularly active against the other two test organisms.

DISCUSSION

The considerable differences found in these studies in degree of cross-resistance between triadimenol and some other DMI fungicides have both practical and theoretical implications. Current strategies for minimising the risk of resistance arising in field populations of pathogens include the avoidance of repeated applications of fungicides having a common mode of action especially when, as with the DMIs, that action is site specific. Since so many fungicides belong to the DMI group, this policy sometimes leaves few suitable alternatives. The possibility of using another DMI fungicide which exhibits a low level of cross-resistance as a substitute for a widely-used product of this type which is losing its effectiveness, might well be considered attractive, but such a policy carries risks. Certain pathogens, notably the powdery mildews, are very sensitive to DMI fungicides and even forms of such pathogens showing a high resistance factor may well remain adequately controlled by the application of normal field rates.

Demonstration of cross-resistance between two fungicides is often taken as evidence for a common mode of action but may not necessarily be so. Unless it is known that resistance to both fungicides arises from the same gene mutation, the cross-resistance could arise from the accumulation, within the same organism, of several mutations governing resistance (Georgopoulos 1982). Alternatively a single gene mutation may be pleiotropic, causing several unrelated changes in the mutant which independently affect its sensitivity to compounds having quite different modes of action (van Tuyl 1977).

There is good evidence (Langcake et al. 1983) that all fungicides classified as DMIs, that have been examined, do indeed inhibit C-14 demethylation. Two of the three triadimenol-resistant isolates examined in these studies showed cross-resistance to all other DMI fungicides. The variations in cross-resistance to the different compounds might result from subtle differences in the precise mechanism by which the fungicides interfere with the demethylation process. There was some correlation between cross-resistance levels and chemical structure. Fenarimol and nuarimol always gave RF values higher than those for triadimenol, whilst those for prochloraz were consistently lower.

Differences in RF values could also arise from differences in uptake and metabolism. It has been established (Deas et al. 1984) that the sensitivity of fungi to triadimefon is frequently correlated with their ability to reduce this compound to triadimenol and with the isomeric composition of the triadimenol produced. It is interesting that the triadimenol-sensitive isolate of P. teres was much less sensitive to tradimefon than to triadimenol, whereas the triadimenol-resistant isolate

showed similar sensitivity to both fungicides.

Some fungicides have additional modes of action. Sud & Feingold (1981) have shown that the imidazole antimycotics, clotrimazole and miconazole, are fungistatic at low concentrations, and cause an accumulation of methylated sterols, whilst at higher concentrations both are fungitoxic, probably through a direct effect on cell membrane permeability.

Chloraniformethan lacks a N heterocycle in its molecule. It exhibited no cross-resistance with known DMIs and, presumably, has a different mode of action. Its molecule does, however, have some similarity to that of triforine (Fig. 1) and this illustrates the danger of assigning a common mode of action to two compounds solely on the basis of structural similarities, if the active grouping within the molecule is not known.

The different pattern of cross-resistance shown by the triadimenol-resistant isolate of P. teres, compared with those of S. fuliginea and C. cucumerinum, could arise from differences in their mechanism of resistance. Van Tuyl (1977) showed that resistance to imazalil is multigenic, which could indicate that this compound has more than one mode of action, or that more than one resistance mechanism is operating. There is evidence that resistance in Aspergillus nidulans to both fenarimol (de Waard & van Nistelrooy 1980) and imazalil (Siegel & Solel 1981) results from reduced accumulation of the fungicide. Resistance to DMIs in a mutant of Ustilago maydis was associated with changes in the composition of its sterols (Walsh & Sisler 1982).

Clearly more information is required before the reason(s) for the differing levels of cross-resistance shown within the DMI group of fungicides can be clarified. Studies on the uptake and metabolism of triadimenol by the isolates of C. cucumerinum, and analysis of fungal sterols, are currently being undertaken by the author. Further investigations into the lack of cross-resistance to many DMI fungicides shown by the triadimenol-resistant isolate of P. teres are in progress.

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CROSS-RESISTANCE RELATIONSHIPS BETWEEN THE BENZIMIDAZOLE FUNGICIDES, N-PHENYL CARBAMATES AND OTHER RELATED COMPOUNDS

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ABSTRACT

The sensitivity of several benomyl-resistant isolates of Fusarium oxysporum, Botrytis cinerea and Venturia inaequalis to different antimitotic agent and related compounds was tested. Cross-resistance between benomyl and N-phenyl carbamates, hitherto considered to be negatively correlated, was found to be dependent on the fungal isolates and species used. Positively correlated cross-resistance also occurred, especially in isolates with a low degree of resistance to benomyl. Increasing the number of chlorine atoms in the phenyl moiety of the carbamates shifts the correlation in a negative direction. Among the isolates of B. cinerea and V. inaequalis the sensitivity to MDPC was correlated with the degree of their resistance to benomyl. Both benomyl-sensitive and -resistant isolates of F. oxysporum were totally insensitive to MDPC, although they did not taken up less, or metabolise more, of this compound as compared with B. cinerea. Other compounds, chemically related to phenyl-carbamates, were also tested and their activity against benomyl-resistant isolates varied greatly. Due to the specific character of the negative cross-resistance observed, it seems unlikely that a compound with general ability for selective control of benomyl-resistant isolates will be found.

INTRODUCTION

The phenomenon of cross-resistance within the benzimidazole fungicides is well investigated and experiments with other antimitotic agents have been carried out in the last decade: Leroux and Gredt (1979, 1982) reported that some benzimidazole-resistant isolates of Botrytis cinerea and Penicillium expansum showed negatively correlated cross-resistance to N-phenyl carbamates but no cross-resistance was observed for Aspergillus nidulans isolates. Kato et al. (1984) demonstrated that a new phenyl carbamate, MDPC, methyl-N-(3,5-dichlorophenyl) carbamate was much more active against benzimidazole-resistant isolates of different fungal species than against wild-types. Similar results were obtained by Gullino and Garibaldi (1986) with B. cinerea using another phenyl carbamate, S 32165. Enisz (1986) demonstrated negative correlation between sensitivity to benzimidazoles and MDPC in isolates of B. cinerea and Gerlachia nivalis highly resistant to benomyl but not in moderately resistant ones. In field experiments the efficacy of MDPC against benomyl-resistant Pseudocercosporella herpotrichoides in wheat was found to be inadequate (Fehrman and Franke 1986). In contrast to the findings reported above weak positive cross-resistance between benomyl and propham, a phenyl carbamate herbicide, was shown in field isolates of Venturia inaequalis (Gasztonyi and Josepovits 1981). Higher sensitivity to benomyl-resistant isolates to N-phenyl carbamates is not, therefore, always observed but seems to be dependent on the fungal species.

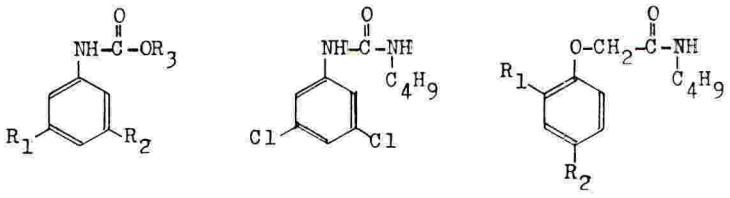
In the studies reported on here we have investigated the level of cross-resistance between benzimidazole fungicides and N-phenyl carbamates shown by various fungal species and isolates. Sensitivity of several fungal species

to both groups of antimitotic agents was also compared. Experiments with derivatives of other antimitotics were also undertaken following an earlier report on the colchicine-like activity of some amide derivatives of 3,5-dichloro-phenoxyacetic acid (Südi and Matolcsy 1962).

MATERIALS AND METHODS

Chemicals

Benomyl was obtained from Chinoin Chemical and Pharmaceutical Works (Hungary), Propham (IPC) [isopropyl-N-phenyl carbamate] (Fig.1; I, R_1 = R_2 = H, R_3 = $CH(CH_3)_2$) and chloropropham (CIPC) [isopropyl-N-(3-chlorophenyl) carbamate] (Fig. 1; I, R_1 = Cl, R_2 = H, R_3 = $CH(CH_3)_2$) from North Hungarian Chemical works, (MDPC) [methyl-N-(3,5-dichlorophenyl) carbamate] (Fig. 1; I, R_1 = R_2 = Cl, R_3 = CH_3) was donated by Research Institute of Plant Protection Kleinmachnow (GDR), BDPU [N-butyl-N'-(3,5-dichlorophenyl) urea] (Fig. 1; II), o-CPAB [N-butyl-2-chlorophenoxy-acetamide] (Fig. 1; III, R_1 = Cl, R_2 = H), p-CPAB [N-butyl-4-chlorophenoxy-acetamide] (Fig. 1; III, R_1 = H, R_2 = Cl) were prepared in our institute. Full details will be published elsewhere. All chemicals used were of analytical grade.



I II III

Fig. 1. Structural formulae of N-phenyl carbamates (I), N-phenyl urea (II) and phenoxy-acetamides (III).

Fungi

Wild (V12) and benomyl-resistant field isolates (V1, V2, V7, V8) of V. inaequalis were provided by S. Rozsnyai (this Institute) and have been maintained on potato dextrose agar (PDA) in our laboratory since 1980. Their resistance factors have been systematically checked. One benomyl-sensitive (F10) and two genetically different benomyl-resistant (ben1 and ben2) mutants (F13, F14) of F. oxysporum, as well as the benomyl-sensitive (F19) and -resistant (F20) recombinants of the ben1- and ben2-type mutants were supplied by L. Hornok (this Institute). For details of isolation see in Molnár et al. (1985). The wild (BS) isolate, and the benomyl-resistant field isolate (BR), of B. cinerea were kindly provided by T. Kaptás (Plant Protection and Agrochemical Station, Eger, Hungary). All other fungal species tested were taken from the collection maintained in this laboratory.

Sensitivity tests

For most test fungi (exept F. oxysporum) fungitoxicity was measured by the following method. Agar discs containing fungal inocula were placed on filter paper discs (20 mm diam.) previously impregnated with solutions in acetone or ethanol of the compound under study and incubated in Petri dishes at a temperature optimal for fungal growth. The impregnation of sterile paper

discs was carried using a series of concentrations. Sufficient molten agar medium, inoculated with a dense suspension of dispersed inoculum, was poured into Petri dishes to make a layer 2 mm thick, from which agar discs of 20 mm diameter were cut out and immediately placed on the impregnated paper discs. After incubation for 4-6 days the growth of fungus was estimated. Due to the small thickness of agar discs the effect of differences in the diffusibility of the tested compounds is negligible, which is an advantage when different substances and different fungal species are compared.

Due to the slow growth of F. oxysporum mutants in the agar disc test, the sensitivity of this fungus was tested by measuring the growth of fungal colonies on agar amended with fungicide. Sensitivity was expressed as the MIC value, this being the lowest dose tested which gave complete inhibition of fungal growth.

Investigation of uptake and metabolism of MDPC

The uptake and metabolism of MDPC in mycelia of F. oxysporum and B. cinerea was measured by incubating the compound with mycelial mats (c. 100 mg) which were placed in 20 ml of potato dextrose liquid medium containing 2 mg MDPC and incubated on a shaker for 1 day at 25°C. Control mats were incubated in the absence of MDPC. After filtration mycelia and culture filtrates were extracted separately with aceton and chloroform respectively. The extracts were concentrated to 2 ml and analysed by glc (column packed with 3% SE-30 on Anachrom ABS 90/100 mesh; temp. 130°C; FID). The recovery of MDPC was established by adding known amounts of the compound separately to mycelium and culture filtrates and the data obtained after their extraction were used to correct levels of MDPC found in the extracts made after incubation.

RESULTS

Cross-resistance between N-phenyl carbamates and benomyl

The minimum inhibitory concentration (MIC) values of N-phenyl carbamates obtained for various isolates of V. inaequalis, F. oxysporum and B. cinerea are summarised in Table 1. MIC values for benomyl are also presented.

In contrast to V. inaequalis and B. cinerea, the benomyl-resistant isolates of F. oxysporum were insensitive to MDPC.

Metabolism experiments

To investigate possible reason for the lack of activity of MDPC to benomyl-resistant isolates of F. oxysporum, the uptake and metabolism of the compound by both benomyl-resistant and -sensitive isolates of B. cinerea and by benomyl-resistant F. oxysporum (F20) were compared (Table 2).

MDPC accumulation in mycelium of benomyl-resistant F. oxysporum was not lower than that in mycelium of B. cinerea isolates both sensitive and resistant to benomyl. Decrease of total amount of MDPC extracted from the isolates of B. cinerea indicates some metabolic transformation (Table 2). Dichloroaniline, a probable metabolite, was not detected in any isolate.

TABLE 1

Comparison of antifungal activity[a] of N-phenyl carbamate and benomyl to isolates of Venturia inaequalis (V), Fusarium oxysporum (F) and Botrytis cinerea (B)

Isolate	Propham	Chloropropham	MDPC	Benomyl[b]
V12	300	30	100	0.05
V1	300	100	20	10
V7	100	10	1	300
V8	100	100	1	1000
F10	150	100	>1000	2
F13	200	100	>1000	10
F14	150	100	>1000	15
F19	150	100	>1000	3
F20	200	100	>1000	100
BS	100	300	200	0.03
BR	100	30	1	3000

[a] MIC (minimum inhibitory concentration), µg/ml
[b] MIC of benomyl for Fusarium isolates are taken from Molnar et al.(1985).

TABLE 2

Uptake of MDPC from a liquid medium by mycelia of isolates of Botrytis cinerea (BS, BR) and Fusarium oxysporum (F20)

Isolate	Dry wt of mycelium (mg)	MDPC extracted			
		Mycelium (µg/mg)[a]	Accumulation[b]	Total (mg)	(as % of added)
BS	134	8.66	33	1.68	84
BR	131	7.98	28	1.62	81
F20	88	13.48	34	1.98	99

[a] Dry wt
[b] Concentration of MDPC found in mycelium (µg/g wet wt)/conc. in culture filtrate (µg/ml).

Investigation of cross-resistance to N-butyl-chlorcphenoxy-acetamides (o-CPAB, p-CPAB) and N-3,5-dichlorophenyl-butyl-urea (BDPU) shown by benomyl-resistant isolates

The fungitoxicity of the compounds tested varied greatly with fungal species. The two acetamides have no activity against F. oxysporum isolates and BDPU was inactive against B. cinerea and F. oxysporum. Negatively correlated cross-resistance between benomyl and BDPU was considerable only with the V7 isolate of V. inaequalis, the later being totally inhibited by BDPU at 10 mg/l, whilethe benomyl-sensitive V12 isolate was growing even in the presence of 1000 mg/l. In the case of o-CPAB, and especially of p-CPAB, positive cross-resistance with benomyl was observed in B. cinerea. MIC of p-CPAB for the benomyl-sensitive isolate of B. cinerea was 10 mg/l, while for the benomyl-resistant isolate it was 100 mg/l.

Activity of N-phenyl carbamates against fungal species with different levels of sensitivity to benomyl

Because of the relationship between the sensitivities of different isolates of V. inaequalis and B. cinerea to benomyl and N-phenyl carbamates, the possibility of similar relationships among fungal species was also studied (Table 3).

The data in Table 3 show that the known insensitivity of the Peronosporales to benomyl is generally associated with a relatively high sensitivity to N-phenyl carbamates. No correlation was observed for other fungal species.

TABLE 3

Comparison of the sensitivity[a] of fungi to N-phenyl carbamates and benomyl

Fungus	Propham	Chloropropham	MDPC	Benomyl
Stemphylium radicinum	1000	300	>1000	>100
Cladosporium cucumerinum	30	100	300	0.1
Botrytis cinerea	100	300	200	0.03
Fusarium oxysporum	150	100	>1000	3
Venturia inaequalis	300	30	100	0.05
Stereum purpureum	1000	30	30	>100
Rhizoctonia solani	>1000	30	300	10
Pythium ultimum	100	30	>1000	>100
Phytophthora cactorum	100	100	100	>100
Phytophthora megasperma	100	30	100	>100

[a] MIC (minimum inhibitory concentration), /ug/ml

DISCUSSION

It has been shown in experiments with V. inaequalis (Gasztonyi et al., unpublished), and with F. oxysporum (Gasztonyi et al., 1986), that benomyl resistance is connected with changes in tubulin structure. Although the site of action of N-phenyl carbamates assumed to be different from that of benzimidazole fungicides, increased sensitivity to N-phenyl carbamates seems to be

a frequent, but not inevitable, consequence of the altered tubulin structure.

As shown in Table 1 the sensitivity of benomyl-resistant strains of V. inaequalis and B. cinerea, but not F. oxysporum, to N-phenyl carbamates, and thus the degree of negative cross-resistance, increased with the number of chlorine atoms in the phenyl moiety. This relationship is illustrated in Fig. 2.

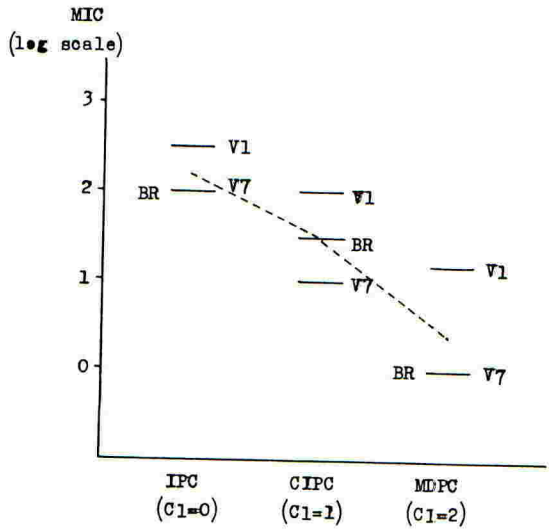


Fig. 2. Relatioship between the number of chlorine atoms in the phenyl moiety of N-phenyl carbamates and their activity (minimum inhibitory concentration) against benomyl-resistant fungi. The dotted line joins the average levels of activity against the three isolates.

The cross-resistance between benomyl and MDPC was found to be negatively correlated in all isolates of V. inaequalis and B. cinerea but not those of F. oxysporum. With propham and chloropropham a slight positive cross-resistance was observed with some isolates.

Lack of sensitivity towards MDPC in F. oxysporum as compared to B. cinerea could not be explained either by a reduced uptake or increased metabolic detoxification (Table 2). Lack of sensitivity to MDPC in all isolates of F. oxysporum might be attributable to either a different structure, or different mode of assembly, of its tubulin.

In benomyl-resistant isolates of V. inaequalis and B. cinerea the increased sensitivity to MDPC shows a linear correlation with the degree of their resistance to benomyl when plotted on a log/log scale (Fig. 3). No significant correlation was obtained for propham and chloropropham.

Among the other compounds investigated (o-CPAB, p-CPAB and BDPU) examples of positive or negative cross-resistance with benomyl were also observed, but their appearance was even more restricted than that for MDPC. It would seem that the genetic changes resulting in benomyl resistance alter the sensitivity of the mutant not only to phenyl carbamates but also to other, related compounds.

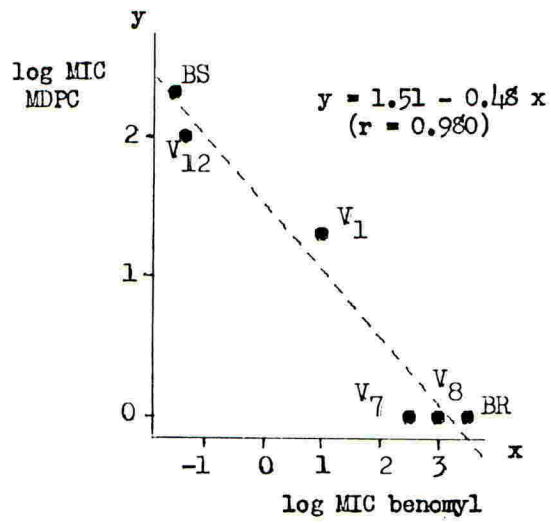


Fig. 3. Correlation between sensitivity to MDPC and to benomyl of fungal isolates.

It may be concluded that although negative cross-resistance between benomyl and other types of antimitotics commonly occurs, the discovery of an agent generally effective against benomyl-resistant fungi is not to be expected.

The comparison of sensitivity of different species of fungi to benomyl and to phenyl carbamates revealed the uniform response of members of the Peronosporales whose insensitivity to benomyl is associated with a relatively high sensitivity to N-phenyl carbamates. This is a further indication of structural differences either in their tubulins or in organising apparatus of microtubuli relative to other fungal species, previously assumed simply on the basis of their natural tolerance to benzimidazoles.

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INHERITANCE OF METALAXYL RESISTANCE IN THE POTATO LATE-BLIGHT FUNGUS

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ABSTRACT

The majority of F1 progeny from crosses between metalaxyl-resistant and -sensitive field isolates of Phytophthora infestans, from the Netherlands and Egypt respectively, were intermediately sensitive to metalaxyl in vitro. F2 progeny from a sib-mating between intermediately sensitive phenotypes segregated in a ratio close to 1:2:1 for sensitive:intermediately sensitive:resistant phenotypes. A 1:1 ratio of sensitive:intermediately sensitive phenotypes was observed in a related backcross to the sensitive parent. Segregation of all three phenotypes occurred among sexual progeny of an intermediately sensitive self-fertile culture from the backcross generation but not among progeny of a self-fertile culture resistant to the fungicide. These data are consistent with resistance to metalaxyl in P. infestans being controlled by a single nuclear locus exhibiting incomplete dominance.

INTRODUCTION

Isolates of Phytophthora infestans resistant to the acylalanine fungicide metalaxyl (N-(2,6-dimethyl phenyl)-N-methoxyacetyl alanine) were detected in 1980 in fungicide-treated potato crops in Eire and the Netherlands (Dowley & O'Sullivan 1981, Davidse et al. 1983).

Recently high levels of oospore germination and establishment of single-oospore cultures of P. infestans have been achieved (Shattock et al. 1986b). Using these techniques the inheritance of metalaxyl resistance in the late-blight fungus was investigated.

MATERIALS AND METHODS

Cultures

Metalaxyl-resistant isolates (1098, 1099 and 1100) (A1 mating type) originated in metalaxyl-treated potato crops in the Netherlands and the metalaxyl-sensitive isolate (isolate E17c) (A2 mating type) was isolated from blighted tubers imported into the U.K. from Egypt in 1984 for public consumption (Shaw et al. 1985).

Establishment of progeny

Single-zoospore cultures of A1 and A2 parents were cultured together on modified 10% clarified V8 juice agar (Shattock et al. 1986a) without illumination. Oospores developed at the hyphal interface of A1 and A2 cultures. Pieces of agar containing 10-20 d old oospores were fed to water snails and after 24-48 h faecal pellets containing undigested oospores were collected, concentrated by sedimentation, homogenised and the oospore suspension surface sterilized with 30 µg/ml $HgCl_2$ for 5 min. Treated oospores were spread over distilled water agar and incubated at 18°C with blue and white light (Shattock et al. 1986a). Germination usually commenced 3-5 d later and single-oospore cultures were established by transferring germinated oospores to either nutrient broth or agar.

Characterization of progeny

Sensitivity to metalaxyl was determined in parental and progeny cultures by comparing the radial growth of each isolate after 8 d incubation at 18°C on 10% clarified V8 juice agar with and without 10 µg/ml a.i. technical grade metalaxyl. Metalaxyl-sensitive, -intermediately sensitive and -resistant phenotypes were defined arbitrarily as exhibiting <10%, >10<60% and >60% growth respectively on metalaxyl-amended agar relative to growth on metalaxyl -free agar. Inocula consisted of agar plugs containing mycelium and four different isolates were placed equidistantly at the edge of each of two plates of unamended and two plates of fungicide-amended V8 agar.

The mating type of single-oospore progeny was determined by pairing each progeny culture with known A1 and A2 cultures and recording either the presence or absence of oospores at the hyphal interface between the isolate and the A1 and A2 cultures. Pure cultures were examined for adpressed growth and the presence of oospores which would indicate self-fertility.

RESULTS

The Egyptian A2 isolate E17c showed less than 5% growth on metalaxyl-amended agar compared with growth on V8 agar alone whereas isolates 1098, 1099 and 1100 were largely unaffected by the fungicide and exhibited growth rates of between 80 and 90% of control. The majority of F1 progeny from the three crosses 1098 x E17c, 1099 x E17c and 1100 x E17c were intermediately sensitive having mean values for growth (relative to control) on metalaxyl-amended agar of 25%, 27% and 28% respectively. In each cross there were also some resistant and sensitive phenotypes amongst the progeny and in each the distribution was skewed towards the sensitive parent, as illustrated in Fig. 1 for cross 1099 x E17c.

There were approximately twice as many A1 as A2 mating types in each F1 generation and c. 12% of the progeny were self-fertile in crosses 1099 x E17c and 1100 x E17c (Table 1).

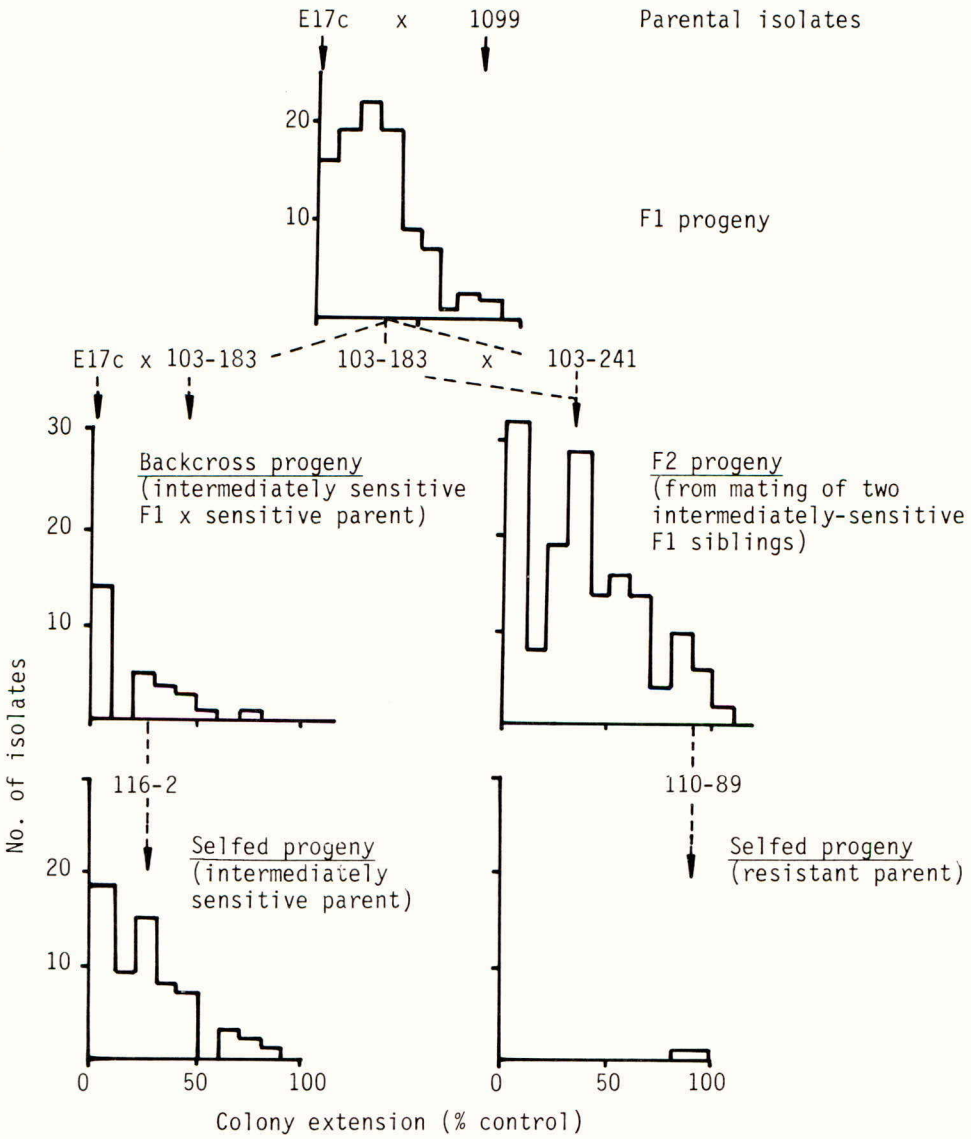
A sib-mating between two F1 progeny of cross 1099 x E17c of identical intermediate sensitivity to metalaxyl produced 154 single-oospores cultures (Fig. 1). The relative growth rates of these F2 progeny in the presence of metalaxyl varied from 0 to 103% of growth on unamended V8 agar. Thirty-two of the progeny were sensitive, 85 were intermediately sensitive and 37 classified as resistant phenotypes (Fig. 1).

Of these 154 F2 progeny, 83 were A1 and 64 A2 mating type while 8 were self-fertile (Table 1).

Viable progeny were also established when the A1 parent of the sib-mating (viz. isolate 103-183) was backcrossed to the metalaxyl-sensitive parent E17c. Fourteen of the progeny were sensitive to metalaxyl, 13 intermediately sensitive; a single isolate was resistant (Fig. 1). A1 and A2 mating types occurred in a 1:1 ratio and two of the progeny were self-fertile (Table 1).

Oospores of one of these self-fertile isolates (116-2) were successfully germinated and of the 63 single-oospores cultures established 18 were sensitive, 39 intermediately sensitive and 6 resistant to metalaxyl. Two single-oospore cultures were also established from a resistant self-fertile isolate of the F2 generation and like their parent 110-89 they were both highly resistant to the fungicide (Fig. 1).

Fig. 1. Distribution of metalaxyl sensitivity* within F1, F2, backcross and selfed generations, resulting from the crossing of metalaxyl-sensitive and -resistant isolates of Phytophthora infestans.



* Sensitivity measured by growth on V8 agar containing 10 µg/ml metalaxyl expressed as % of growth on unamended agar.

In each case progeny of these self-fertile isolates segregated for mating type (Table 1).

TABLE 1

Mating type of single-oospore cultures of F1, F2, backcross and selfed generations from matings between metalaxyl-resistant and -sensitive parental isolates of Phytophthora infestans

Generation	A1 parent	A2 parent	No. progeny of mating type		
			A1	A2	Self-fertile
F1	1098	E17c	23	10	0
	1099	E17c	63	36	12
	1100	E17c	25	13	5
F2	103-183[a]	103-241[a]	83	64	8
Backcross	103-183[a]	E17c	13	13	2
Selfed	110-89[b]	self-fertile	1	1	0
	116-2[c]	self-fertile	28	33	2

[a] F1 progeny from cross 1099 x E17c

[b] F2 progeny from cross 103-183 x 103-241

[c] Backcross progeny from cross 103-183 x E17c

DISCUSSION

The majority of F1 progeny were not parental phenotypes for metalaxyl sensitivity but instead were intermediate in their response to the fungicide. This observation, the segregation of sensitive, intermediately sensitive and resistant phenotypes in F2 close to a 1:2:1 ratio (χ^2 = 1.987, P = 0.35) and the 1:1 segregation for sensitive to intermediately sensitive phenotypes in the backcross to the metalaxyl-sensitive parent, all suggest that metalaxyl resistance is controlled by a single nuclear locus exhibiting incomplete dominance. Thus metalaxyl-resistant and -sensitive isolates are homozygous whereas the -intermediate phenotype is heterozygous. Further evidence to support this single gene hypothesis is provided by the sexual progeny of the self-fertile isolates. Segregation was observed among single oospore progeny of the intermediately sensitive (presumably heterozygous) isolate 116-2 but not among the two single-oospore cultures from the metalaxyl-resistant isolate 110-89.

Some parental phenotypes occurred among the F1 progeny and these are likely to be selfs. Selfing by parents occurs during normal matings and is readily detected by isozyme analysis if parents are homozygous for different alleles at polymorphic loci such as glucosephosphate isomerase 1 (GPI-1) (Shattock et al. 1986b). In a cross between isolate 1100, used in this study, and isolate 550 from Mexico (Tooley et al. 1985) c. 10% of the F1 progeny were selfs and either sensitive or resistant to metalaxyl (Shattock, Sweigard

& Fry, unpublished data). Unfortunately, the Dutch and Egyptian parental isolates described here were identical homozygous genotypes at the GPI-1 locus.

Equal ratios of A1:A2 are expected among sexual progeny of heterothallic species of Phytophthora (viz. F2 and backcross generations) because the A1 mating type is considered homozygous recessive (aa) and A2 heterozygous (Aa). Selfing by either parent during normal mating could account for the unequal ratios of mating type exhibited by the F1 generations (Table 1). It is not unusual, however, for a large excess of one mating type to occur among sexual progeny even when selfing can be discounted by isozyme analysis (Shattock et al. 1986b). Self-fertile isolates are not uncommon among sexual progeny of P. infestans. In related heterothallic species self-fertile isolates are trisomic involving a duplication of a chromosome of the translocation complex carrying the mating type determinants (Shaw 1983). They are presumably either AAa or Aaa genotypes and consequently can give rise to A1, A2 and self-fertile progeny (Table 1).

The inheritance of metalaxyl resistance in P. infestans appears similar to that described by Crute et al. (1985) for Bremia lactucae. As in the case of B. lactucae the fungicide-resistant parental isolates of P. infestans were isolated from crops treated with metalaxyl. The genetical mechanisms by which metalaxyl-resistant isolates arose in field populations of P. infestans can only be surmised. In asexually reproducing populations mutation of chromosomal genes in a diploid fungus would give rise to heterozygotes intermediately sensitive to metalaxyl. Further mutation, or alternatively crossing-over during mitotic prophase in heterozygous diploid nuclei, would produce homozygous segregants exposed to selection by the fungicide. Meiotic recombination and segregation would achieve the same result either by selfing or hybridization (Shattock et al. 1986a & b) and cannot be excluded since A2 mating type isolates have been reported recently from several European countries including the United Kingdom and have been identified in field isolates collected as early as 1981 in North Wales (Tantius et al. 1986).

The mode of action of acylalanines is compatible with the evidence presented here for a single gene controlling resistance to metalaxyl. Metalaxyl affects nucleic acid synthesis (Davidse et al. 1983) and the target enzyme is RNA polymerase. Presumably heterozygous genotypes produce both metalaxyl-sensitive and -resistant enzyme species and therefore, in the presence of the fungicide, exhibit growth rates typified by the intermediately-sensitive phenotype.

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RECOMBINATION AS A MEANS OF PREDICTING FUNGICIDE RESISTANCE IN BARLEY POWDERY MILDEW

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ABSTRACT

Satisfactory tests for predicting the development of resistance are required by chemists seeking to develop novel fungicides. Such tests must attempt to identify the range of variation in sensitivities that exists for any new compound, and upon which selection might then act. This paper compares the extent of the variation revealed by monitoring "base-line" populations, by mutation, or by recombination, for four fungicides used against barley powdery mildew (Erysiphe graminis f.sp. hordei). Recombination yielded the greatest variation to both ethirimol and triadimenol, fungicides to which resistance has developed in field populations. Recombination did not generate any greater resistance to fenpropidin than that found in field populations, whilst no significant variation was generated to fenpropimorph by any method.

INTRODUCTION

Use of many modern fungicides has been curtailed by the development of resistance. Good predictive tests are, therefore, required to enable synthetic chemists to assess the risk of resistance to any new group of fungicides they might discover. Information on the available genetic variation, and its response to selection, must be obtained at an early stage in development of a new compound. Where significant variation in fungicide sensitivity is encountered, the eventual outcome of using a new fungicide will depend on the nature of the genetic control of this variation, its heritabilty and the fitness of resistant isolates.

The range of variation in wild-type populations has generally been measured either by monitoring, or by selection of mutants following treatment with ultra violet light or chemical mutagens. Where appropriate genetic systems exist, the generation of recombinants offers a third way to examine available variation, especially where it is likely to be controlled by minor genes (polygenes). This paper compares these three methods as ways to identify variation to aminopyrimidine, triazole and morpholine fungicides in barley powdery mildew (Erysiphe graminis f.sp. hordei).

MATERIALS AND METHODS

Much of the methodology has been described in an earlier paper (Hollomon et al. 1984) and only modifications introduced since then will be outlined here. Cultures from field populations were maintained as single pustule isolates, and no changes in their fungicide sensitivity were detected during several years in culture. Technical grade fungicides were used throughout, and were gifts from their respective manufacturers. Apart from some ethirimol assays which were done on cellophane membranes (Hollomon 1977), all bioassays were conducted using barley leaf segments (cv Proctor) floating on solutions containing

different concentrations of each fungicide. Very variable results were initially obtained with fenpropimorph, probably because of its pronounced vapour phase activity. This vapour effect seemed less critical where fenpropimorph concentrations were not allowed to exceed 0.05 µg/ml and, in this way, variation between assays was considerably reduced. Similar difficulties were not encountered with fenpropidin, which was used in concentrations up to 5 µg/ml.

Attempts were made to select mutants from not only isolate 23D5, which was sensitive to all four fungicides used here, but also N42/9 which was itself derived from 23D5 after N-methyl-N'-nitro-N-nitrosoguanidine (NTG) treatment. An ethirimol resistant isolate DH14 (Hollomon 1981), collected from the field in 1976, was used in some mutagenesis experiments.

Crosses between isolates were carried out in a Fisons 600-H growth cabinet using the environmental conditions described previously by Hollomon et al. (1984) ie. 16h photoperiod, 70% relative humidity, and 20°C until cleistothecia were observed. Seedlings (cv Proctor) were grown for 3 weeks in soil-less compost (Arthur Bowers Ltd., Lincoln, UK.), and the second leaf then inoculated with 20 µl of a mixed conidial suspension of both parental isolates in the fluorocarbon FC 43 (Minnesota, Mining and Manufacturing Ltd., Manchester UK.). This suspension (final concentration 10 mg conidia/ ml) contained equal quantities of conidia from each parental isolate.

RESULTS

Variation in natural populations

Bioassay data has been collected over the past 12 years for many single pustule isolates. Table 1 summarises results for four different fungicides for isolates obtained from throughout the UK. Isolates assayed for ethirimol or triadimenol sensitivity were collected before any widespread decline in the performance of these two fungicides was observed in the field. No such decline in the field performance of fenpropidin or fenpropimorph has yet been observed. Consequently, data probably represent "base-line" sensitivities for all four fungicides. Although the number of isolates monitored was small in relation to the total barley mildew population, significant variation in sensitivity was, nevertheless, detected to ethirimol, triadimenol and fenpropidin, but not to fenpropimorph.

TABLE 1

Fungicide sensitivity in natural populations of barley powdery mildew

Fungicide	Range of ED50 (µg/ml)	LSD (5%) between two isolates	Number of isolates tested
Ethirimol	0.003 - 0.098	0.032	35
Triadimenol	0.001 - 0.142	0.025	90
Fenpropidin	0.031 - 1.25	0.155	25
Fenpropimorph	0.0001- 0.024	0.030	94

Variation induced by mutation

Mutagenesis of 23D5 with NTG has so far only yielded two mutants with altered fungicide sensitivity. In addition to the mutant (N91) showing a small decrease in triadimenol sensitivity reported previously (Hollomon et al. 1984), a second mutant (N42/9) has been generated which is less sensitive to ethirimol. In greenhouse tests N42/9 infects equally barley seedlings grown from untreated seed, and from that treated with ethirimol at 12.5% of the commercial dose rate; it is less sensitive than its parent on leaf segments (S.P. Heaney, personal communication). In laboratory tests its resistance is primarily expressed during colony growth and not during appressorial development (Table 2). This contrasts with all ethirimol resistant field isolates so far examined, which differ from wild-type sensitive isolates in that they appear to withstand higher ethirimol doses only during appressorial formation (Hollomon 1977). Attempts to generate further decreases in fungicide sensitivity by mutagenesis of N91 or N42/9 or of field isolates already resistant to ethirimol or triadimenol, have not yet been successful. In parallel work, however, several mutants with altered virulence characteristics have been produced.

TABLE 2

Ethirimol sensitivity of the mutant N42/9

Isolate	Ethirimol sensitivity	Bioassays (ED50)		
		Whole plant (mg/g seed)	Appressorial formation (µg/ml)	Colony length (µg/ml)
23D5	Sensitive	0.25(0.17-0.37)	0.40(0.29-0.55)	1.05(0.75-1.46)
N42/9	Intermediate	1.51(1.30-1.82)	0.54(0.42-0.71)	5.25(3.80-7.24)
DH14	Resistant	Above commercial dose	3.45(2.50-4.76)	1.19(0.86-1.61)

The commercial dose rate is equivalent to 4 mg ethirimol/ g seed. Even at four times the commercial rate infection by DH14 was not reduced by 50%. Procedures for appressoria and colony length bioassays are described elsewhere (Hollomon 1977).
95% Confidence limits are given in parentheses.

Variation generated by recombination

Crosses between either ethirimol or triadimenol sensitive mildew isolates yielded progeny less sensitive to the two fungicides than their parents (Table 3). In both crosses recombination released greater variation than was observed when monitoring base-line sensitivity in natural populations (Table 1), even though fewer progeny were tested than field isolates. However, in neither cross were progeny recovered that were as resistant to triadimenol or ethirimol as those eventually isolated from field populations where disease control was poor. Significant progeny variation was also encountered to fenpropidin, but this was no greater than the variation found when monitoring wild-type populations. No genetic variation was detected in the one cross so far analysed for fenpropimorph sensitivity.

TABLE 3

Variation in fungicide sensitivity generated through recombination

Fungicide	Fungicide sensitivity (ED50 µg/ml)			
	Parental Isolates	Range of sensitivitiy in progeny	LSD(5%) between progeny	Number of progeny
Triadimenol	0.002 0.008	0.007 - 0.420	0.025	30
Ethirimol	0.017 0.030	0.010 - 0.600	0.100	17
Fenpropidin	0.850 0.760	0.040 - 0.910	0.155	16
Fenpropimorph	0.0008 0.0015	No significant variation		31

DISCUSSION

Mutation seems the least effective of the three methods examined for generating variation in fungicide sensitivity. In mutants the mechanism of resistance may also be different from that in resistant isolates collected from the field. Recombination produced significant reassortment of genes for ethirimol and triadimenol sensitivity, and generated greater variation in sensitivity to the two fungicides than was revealed through monitoring natural populations. Fewer progeny were analysed than field isolates indicating that recombination can be an effective way of revealing at least part of the potential variation to a fungicide, especially where variation is controlled by minor genes. Cleistothecia are abundant on ripening barley, and can be stored for years in a refrigerator yet still produce viable ascospores. Ideally more than one cross should be analysed, but progeny may be selected in bulk at appropriate fungicide levels, rather than assayed individually.

The greater variation revealed by recombination was eventually selected for in field populations after repeated use of ethirimol or triadimenol, with the result that their performance declined, especially on mildew suceptible cultivars. The inability to detect genetic variation to fenpropimorph, and the fact that recombination did not generate a greater range of fenpropidin sensitivities than is present in field populations, suggests that insufficient genetic variation to morpholine fungicides may be available in barley mildew to allow selection towards resistance levels that might seriously affect performance.

Mathematical models have been used to predict the likely development of fungicide resistance, and the probable effects of different strategies of fungicide use on its spread. To date, models have effectively described the evolution of fungicide resistance only where it is controlled by a single gene with two alleles. Models dealing with

polygenically controlled quantitative resistance are being developed (Skylakakis & Hollomon 1987), but require measurement of genetically controlled characteristics such as heritability, and the range of variation available for selection ("selection differential", Via 1986). Analysis of sexual progeny not only provides information on heritability but also provides an effective way of determining the possible selection differential. Consequently, data generated through recombination are likely to be essential where models are used to develop strategies of fungicide use which might combat the spread of polygenically controlled resistance.

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