Session 4C

Pest and Disease Resistance to Agrochemicals

Session Organiser — Dr D R Jones

Poster Papers — 4C-1 to 4C-15

EFFECT OF TRIAZAMATE ON RESISTANT *MYZUS PERSICAE* ON SUGAR BEET UNDER FIELD CAGES

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ABSTRACT

Triazamate at 70 and 105 g AI/ha, and at 56 g and 70 g AI/ha plus adjuvant oil gave excellent control of resistant biotypes of *Myzus persicae* on sugar beet under field cages. After two sprays triazamate was almost completely effective, especially when mixed with oil, whereas pirimicarb allowed a few highly resistant survivors, and deltamethrin plus heptenophos and deltamethrin alone gave poor control of even moderately resistant aphids. Triazamate is therefore an excellent potential alternative to existing recommended products, especially where the proportion of highly resistant biotypes is high.

INTRODUCTION

The novel insecticide, triazamate (a carbamyl triazole), was first developed as a persistent, systemic aphicide spray, particularly suitable for dicotyledonous crops (Murray *et al.*, 1988). Assessments of its performance on sugar beet in the laboratory and in the field confirmed its excellent efficacy against *Myzus persicae*, even those which were highly resistant to currently recommended insecticides (Dewar *et al.*, 1988; 1992; Furk & Murray, 1988). In those earlier trials, the rates of triazamate applied were quite high (140-280 g AI/ha) and the formulation was a wettable powder. The experiments described here present a summary of results from three trials conducted over three years with much lower rates of an EC formulation.

MATERIALS AND METHODS

Sugar beet, cvs Celt in 1991, and Saxon in 1992 and 1993, was sown deliberately late, on 15 May in 1991, 18 May in 1992 and 11 May in 1993, to encourage large numbers of aphids to colonise the young plants during their peak migrating period in late June, and thus provide sufficient aphids to assess the efficacy of novel insecticides. To assess the efficacy of insecticides against resistant *Myzus persicae*, 30 plants per plot were infested with aphids from four clones reared in the glasshouse on potted sugar

beet plants; US1L (susceptible-S), 405D (moderately-resistant R_1), T1V (highly resistant, R_2) and 794J (extremely resistant R_3) (Sawicki *et al.*, 1978). Ten aphids were placed on each plant in the ratio $S:R_1:R_2:R_3 = 2:5:2:1$, approximating that found in field samples in recent years. Semi-circular field cages, 0.5m x 3m with a radius of 0.5m, covered with x mesh Protex^R netting, were placed over two rows of 15 plants per plot to minimise natural infestation and limit aphid-specific predators such as ladybirds which are attracted to released populations of aphids. Populations were allowed to build up for 7-14 days before treatments, which were replicated three times in randomised blocks in 1991 and 1993, and four times in 1992. The degrees of freedom in the tables reflect the additional treatments not reported here.

Treatments

Cages were removed only during sampling and spray treatments. Sprays were applied using a two-man Oxford precision sprayer, delivering 260 l/ha through Lurmark 03F 80 nozzles at 2.4 bar in 1991, and 200 l/ha through Lurmark O2F 80 nozzles at 2.0 bar in 1992 and 1993. Triazamate as an EC formulation at 70 (TZ-M) and 105 (TZ-H) g AI/ha in 1991 and 1992, and at 56 (TZ-L) and 70 g AI/ha mixed with adjuvant mineral oil (Swirl) at 500 ml/ha in 1993, was compared with the two currently recommended treatments, pirimicarb (Pir; Aphox) at 140 g AI/ha, and deltamethrin plus heptenophos (D + H; Decisquick) at 7.5 + 120 g AI/ha. Deltamethrin (Delt; Decis) at 7.5 g AI/ha was also compared in 1992 and 1993. All were applied twice, the second application being 6-11 days after the first, depending on the weather.

Assessments

Each trial was sampled five times, initially on the day before the first application, then two days and between 6 and 9 days after each application. On each occasion five plants per plot were cut at ground level, and stored in shallow trays at 4°C to limit movement of the aphids. Plants were examined on the same day in the laboratory and the number of aphids recorded as green or black, winged or wingless. Most were *M. persicae* which had been placed on the plants but some *Macrosiphum euphorbiae* and *Aphis fabae*, were present particularly in 1991, having colonised the plants before caging. Results for *M. persicae* only are presented here. Large *M. persicae* (adults and 4th instars) were placed individually in wells of ELISA-plates containing 50 µl of PBS Tween and stored in a freezer. Smaller aphids were reared to adulthood on leaves of chinese cabbage (*Brassica pekinensis*) in Blackman boxes and frozen in ELISA-plates. The aphids were categorised S, R_1, R_2 or R_3 using the immunoassay described by Devonshire *et al.* (1986).

RESULTS

In 1991 and 1992 both pirimicarb and triazamate at both rates tested gave excellent control (>85%) of resistant aphids throughout (Table 1A & B; Fig. 1 & 2). However, in 1993 triazamate at 70 g AI/ha but not the low rate mixed with oil was significantly better than pirimicarb, 2 and 6 days after the first application; this difference was less noticeable after the second application (Table 1C; Fig. 3). Although

TABLE 1. Effect of aphicide sprays on the number (log n + 1) of resistant *Myzus persicae* on sugar beet under field cages.

A) 1991: Pre-treatment numbers = 9.8 aphids per plant

Treatment	Days after treatment 1		Days after treatment 2	
	2	8	2	7
Untreated	0.777(5.0)*	0.884(6.7)	0.999(9.0)	1.028(9.7)
Pirimicarb	0.060(0.1)	0.120(0.3)	0.092(0.2)	0.200(0.6)
Delt + Hept	0.845(6.0)	0.766(4.8)	1.086(11.2)	1.533(33.1)
Triazamate M	0.050(0.1)	0.112(0.3)	0.080(0.2)	0.401(1.5)
Triazamate H	0	0.136(0.4)	0.040(0.1)	0.156(0.4)
SED (18 d.f.)	0.1047	0.1684	0.1380	0.1647

B) 1992: Pre-treatment numbers = 26.6 aphids per plant

Treatment	Days after treatment 1		Days after treatment 2	
	2	7	2	7
Untreated	1.450(27.2)	1.741(54.1)	1.816(64.4)	1.650(43.7)
Pirimicarb	0.466(1.9)	0.672(3.7)	0.712(4.2)	0.472(2.0)
Delt + Hept	1.117(12.1)	1.337(20.7)	1.221(15.6)	1.139(12.8)
Deltamethrin	1.329(20.3)	1.322(20.0)	1.218(15.5)	1.145(13.0)
Triazamate M	0.358(1.3)	0.499(2.2)	0.715(4.2)	0.305(1.0)
Triazamate H	0.223(0.7)	0.442(1.8)	0.685(3.8)	0.274(0.9)
SED (21 d.f.)	0.1271	0.1141	0.1112	0.1237

C) 1993: Pre-treatment numbers = 11.5 aphids per plant

Treatment	Days after treatment 1		Days after treatment 2	
	2	6	2	7
Untreated	1.195(14.7)	1.298(18.9)	1.223(15.7)	0.880(6.6)
Pirimicarb	0.775(4.7)	0.791(5.2)	0.369(1.3)	0.309(1.0)
Delt + Hept	0.795(5.2)	0.909(7.1)	0.793(5.2)	0.540(2.5)
Deltamethrin	1.011(9.3)	1.020(9.5)	0.937(7.6)	1.033(9.8)
Triazamate L	0.588(2.9)	0.333(1.2)	0.134(0.4)	0.053(0.1)
Triazamate M	0.412(1.6)	0.253(0.8)	0.026(0.1)	0.053(0.1)
SED (22 d.f.)	0.1385	0.1553	0.1324	0.1780

* figures in brackets are back-transformed; L, low; M, medium; H, high rate

FIGURE 1. Effect of insecticides against susceptible ▢, moderately resistant ▢, highly resistant ▩, and extremely resistant ■, *Myzus persicae* on sugar beet: 1991. See text for explanation of labels.

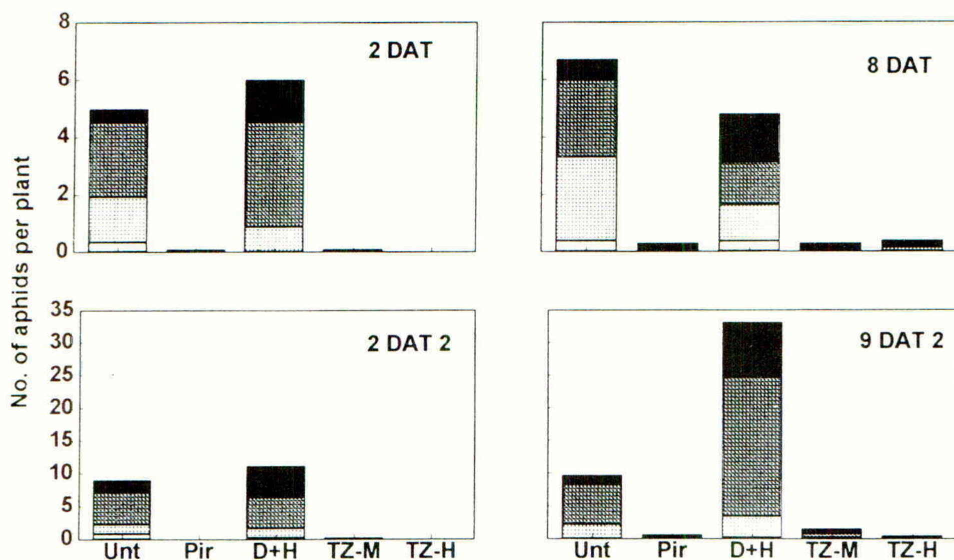


FIGURE 2. As Figure 1 for the year 1992

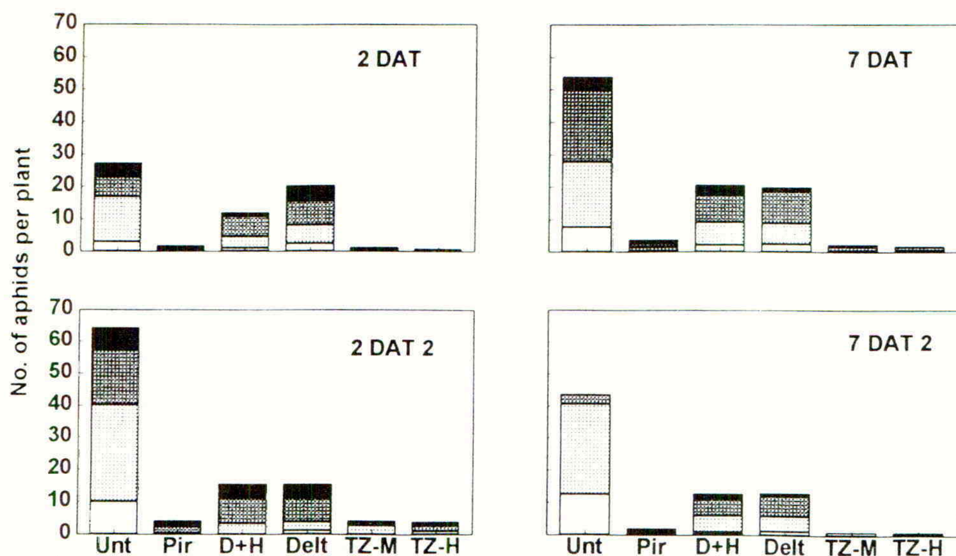
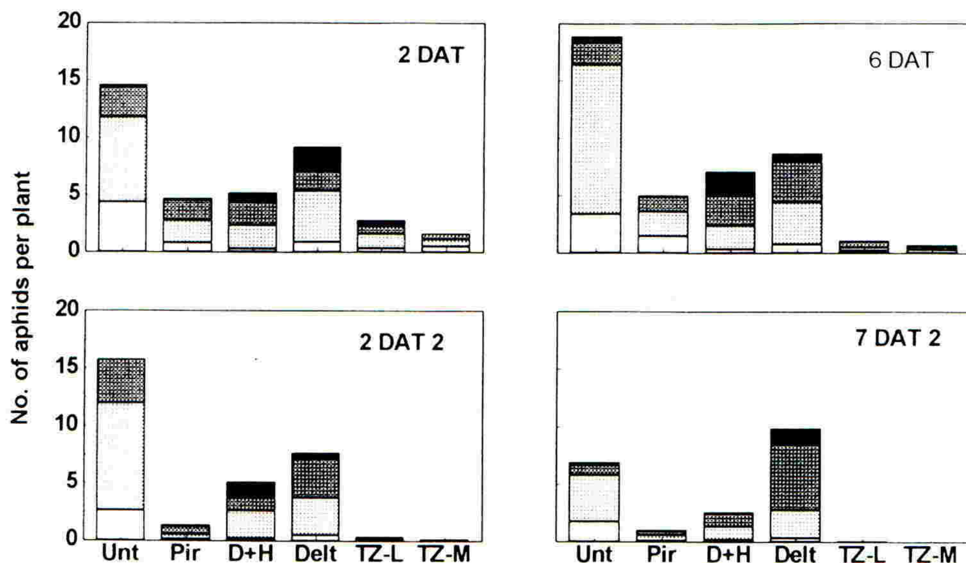


FIGURE 3. As Figure 1 but for the year 1993



most survivors two days after these treatments were R_2 or R_3 aphids, there was no resurgence in later samples. There were virtually no survivors in 1993 after treatment with triazamate plus oil at both rates (Fig. 3). Deltamethrin plus heptenophos gave no control in 1991 and poor control in 1992 and 1993 (max 75%). Indeed in 1991 some resurgence of aphids occurred in plots 7 days after the second application (Fig. 1). Deltamethrin alone performed equally badly in the latter years. These pyrethroid-containing products had no effect on R_2 and R_3 aphids whenever they were applied; only susceptible aphids were efficiently controlled (Figs. 2 & 3).

DISCUSSION

Triazamate at all rates tested gave excellent control of even highly resistant (R_2) and extremely resistant(R_3) *M. persicae*, particularly when mixed with adjuvant oil in 1993. It performed at least as well as pirimicarb against all resistant biotypes, even at the rate of 56 g AI/ha. Although the difference in persistence between pirimicarb and triazamate was not apparent due to the cages preventing re-immigration, in open field trials triazamate gave much longer control (Dewar; unpublished results). Its good performance against R_3 aphids is in contrast to laboratory bioassay results in Australia (Herron *et al.*, 1990) and the UK (Moores *et al*, 1994) and suggests that under field conditions in the UK there are no problems with control of these biotypes. However, some clones of *M. persicae* have developed a new mechanism of resistance to pirimicarb and triazamate, threatening the efficacy of these products. Fortunately, this insensitive-acetylcholinesterase mechanism has not yet been found in the UK (Moores *et al.*, 1994). The poor control of resistant aphids by deltamethrin alone or formulated with heptenophos was typical of pyrethroids. Cypermethrin and cyhalothrin have encouraged rapid increases in *M. persicae* numbers

after two applications (Dewar *et al.*, 1992), and similar adverse consequences of multiple applications have been recorded in potatoes (ffrench-Constant *et al.*, 1988; Harrington *et al.*, 1989).

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INSECTICIDE RESISTANCE DUE TO INSENSITIVE ACETYLCHOLINESTERASE IN *MYZUS PERSICAE* AND *MYZUS NICOTIANAE*

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ABSTRACT

The recent detection of pirimicarb and triazamate insensitive acetylcholinesterase in some populations, in addition to the well-established esterase-based resistance, is likely to influence future tactics for managing insecticide resistance in *Myzus* spp.

INTRODUCTION

Until recently, the only insecticide resistance mechanism identified in the peach-potato aphid, *Myzus persicae* (Sulzer), was based on the overproduction, due to gene amplification, of a carboxylesterase (E4 or FE4) that degrades and sequesters organophosphorus, carbamate and pyrethroid insecticides (Devonshire, 1989). The same amplified genes are also found in the tobacco-feeding form (Field *et al*., 1994), recently classified as a distinct species, *M. nicotianae* (Blackman & Spence, 1992). Due to the relatively low level of resistance to carbamates conferred by this ubiquitous mechanism, they have often acquired a key role in controlling resistant aphids. However, we recently reported (Moores *et al*., 1994) the rare occurrence of resistant strains with a modified acetylcholinesterase (AChE) specifically insensitive to pirimicarb, and to the novel aphicide, triazamate. This gene, even when present only in the heterozygous condition, enhances resistance to these products in bioassays by approximately 15-fold, rendering aphids carrying both the E4 and target site mechanisms effectively immune to these chemicals. We now report the incidence of this mechanism in other populations, including one in which the gene is homozygous.

MATERIALS AND METHODS

Aphids

The origins and rearing of the susceptible (S, clone US1L) and resistant (R_2, clone T1V) reference clones of *M. persicae* and 926B have been described previously (Moores *et al*., 1994). Clone 926B was cloned from aphids collected from tobacco in Greece in 1990 and positively identified as *M. nicotianae* with FE4 levels comparable to R_3 (Devonshire, 1989). Clone 1051A was collected on spinach from Okinawa; it was confirmed as *M. persicae* and has E4 characteristic of R_2 aphids. Clones 1140H, 1173A and 1253Z were established from populations collected in 1993 from peppers in Dutch glasshouses. Both have the A1,3 chromosome translocation characteristic of very resistant aphids containing increased E4, but morphometric analysis did not provide a

clear classification as *M. persicae* or *M. nicotianae*. However, their GOT allozymes (Blackman & Spence, 1992) were characteristic of *M. persicae*. Clone 1200Q was established from a population collected in 1993 from peaches in Argentina, and was identified as *M. persicae* of normal karyotype.

Insecticides

Insecticides used for AChE inhibition studies were of technical grade, with purities greater than 95%. For bioassay, pirimicarb was used as the commercial formulation, Aphox (500 g AI/litre), whilst triazamate was technical grade, both diluted to the appropriate concentrations in 0.01% aqueous Agral.

AChE preparation, assay and inhibition

Approx 250 mg of aphids were homogenised in 2 ml 0.1 M phosphate buffer, pH 7.5, containing 0.1% wt/V Triton X-100, and centrifuged at 1100 g for 5 minutes. The supernatant was then passed through a Sephadex G-25 column (6 cm x 1 cm dia) and then through a procainamide affinity column. After eluting all unbound proteins, the AChE was eluted in 20 mM phosphate buffer, pH 7, containing 1% Triton X-100 and 1M NaCl, with a recovery of better than 80%. The overall degree of purification was not important and therefore not measured, but the removal of other esterases was confirmed by assaying the eluate with 1-naphthyl acetate. This separation of AChE from non-specific carboxylesterases was essential as the large amount of E4 present in the R_3 variants can sequester significant proportions of the insecticide, leaving the target enzyme unexposed to inhibition and giving the appearance of AChE insensitivity *in vitro* (Moores *et al.*, 1994).

AChE activity was assayed with acetylthiocholine at 25°C using a Thermomax microplate reader (Molecular Devices). Preliminary screening of AChE sensitivity to a range of insecticides (or their activated products) was determined by adding the enzyme (0.1 ml) to the wells of microtitre plates containing acetylthiocholine iodide (ATChI, 0.5 mM), 5,5′dithio-bis (2-nitrobenzoic acid) (DTNB, 15 μM) and various concentrations of the inhibitor to give a final volume of 0.3 ml. Activity was monitored continuously for 20 minutes and the mean residual activities used to estimate and compare I_{50} values (the insecticide concentration giving 50% inhibition under the experimental conditions). Only those inhibitors showing different potency to the AChE from various strains were studied in more detail.

Inhibition was measured in the absence of substrate, by mixing inhibitor in buffer with enzyme (50 μl of each) and assaying remaining activity at 10 second intervals by adding 20 μl portions to 100 μl ATChI and 100 μl DTNB to give 0.5 mM and 15 μM final concentrations respectively. The latter very low concentration was not rate-limiting, but was necessary to avoid the inhibition of aphid AChE by DTNB (Moores *et al.*,.1994). Bimolecular rate constants (k_i) were calculated by non-linear regression using Enzfitter software (Leatherbarrow).

K_m values were determined at 25°C by non-linear regression (Enzfitter) of enzyme activity measurements over 2 minutes with ATChI concentrations from 40-640 μM, minimum 10 concentrations for each.

Bioassays

Pirimicarb was bioassayed using the FAO-recommended aphid dip test (FAO, 1979; alternative B) with the dipped aphids being scored for percentage death after 24 h instead of 1 hr. This bioassay regime resulted in insufficient kill when using triazamate, so this compound was bioassayed using excised chinese cabbage leaves in 'Blackman' boxes with their petioles immersed in triazamate solutions for 24 h prior to placing aphids on the leaf surface. The aphids were scored for percentage death after 24 h exposure to the treated leaves still in triazamate solutions. LC50 values for both types of bioassay were calculated by probit analysis using the POLO program (Leora Software).

RESULTS AND DISCUSSION

In preliminary experiments with various inhibitors, I_{50} plots were compared for AChE in homogenates of 1200Q, 1051A and R_2 clones, all having the same E4 levels. The assays showed that, in response to pirimicarb (Fig. 1a), the AChE of 1200Q is homozygous resistant whilst that of 1051A is heterozygous and that of R_2 is homozygous sensitive.

The plateau at around 65% remaining activity indicates that the resistant form of AChE has approximately twice the catalytic centre activity (k_{cat}) of the susceptible enzyme. This greater k_{cat} is also evident in the plots from the microplate assay (Fig. 2) as a higher activity in the wells containing the resistant form of AChE, whether homozygous or heterozygous. All other clones with the sensitive form of the enzyme showed levels similar to that of T1V, whilst those with the insensitive AChE had the higher activity.

Fig. 1 a. Inhibition of AChE by pirimicarb in the presence of substrate. Percent activity is based on the mean activities measured continuously for 20 minutes during these coincubation assays. The top concentration used (3.3 mM) is close to the solubility limit of pirimicarb; b. Inhibition of AChE by 40uM pirimicarb in a preincubation assay.

Fig. 1a.

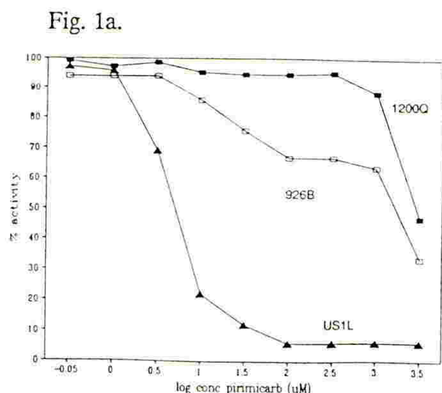

Fig. 1b.

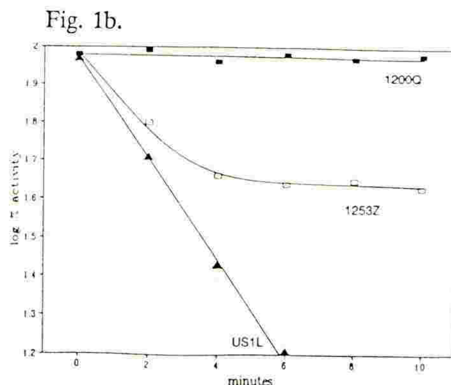

Fig. 2. Plots from microplate assay showing AChE activity in uninhibited (U) wells and wells containing a discrimnating concentration of 17uM pirimicarb (I).

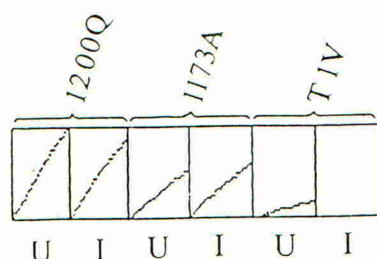

TABLE 1. Michaelis constants (K_m, μM) and bimolecular rate constants (k_i, mM^{-1} min^{-1} $\pm$ s.e.) for inhibition of *Myzus* AChE

Strain	K_m	Pirimicarb	Triazamate
US1L	141 ± 7	193 ± 26	7550 ± 450
926B	221 ± 21	0.63 ± 0.04	554 ± 79
1200Q	227 ± 17	0.91 ± 0.13	449 ± 25
1173A	242 ± 28	0.42 ± 0.12	404 ± 48
1140H	221 ± 31	0.44 ± 0.06	306 ± 18
1235Z	234 ± 26	0.45 ± 0.03	410 ± 36

Preincubation assays with inhibitor also showed the differences in zygosity between the various clones (Fig. 1b). An initial rapid inhibition in the 1173A corresponds to the susceptible component, equivalent to that in the T1V, followed by a slower inhibition corresponding to the insensitive form as found in the 1200Q. The heterozygosity of the AChE in another clone, 926B has been confirmed in genetic crosses (Blackman & Devonshire, unpublished).

The K_m of the insensitive AChE is significantly higher than that of the sensitive enzyme (Table 1), which would be expected to give a smaller "protective effect" (Moores *et al.*, 1994) during co-incubation assays compared with the susceptible enzyme.

Values for k_i were determined using high inhibitor concentrations and short preincubation times to ensure full inhibition of the susceptible component in the heterozygotes from the start of the assay, thus allowing first order inhibition kinetic measurements on the insensitive component. The insensitive enzymes were indistinguishable between 1200Q, 926B, 1140H and 1173A, all showing a marked insensitivity to pirimicarb (> 100-fold) and triazamate (> 10-fold) compared with the

sensitive AChE. Despite the high inhibitor concentrations, there was no significant formation of a Michaelis complex between either enzyme form and inhibitor (Table 1).

Resistance due to this insensitive AChE seems to be restricted to pirimicarb and triazamate, both of which give a dimethylcarbamylated enzyme. In 1051A the presence of the insensitive AChE in a heterozygous form confers resistance to pirimicarb some 14-fold over the clone with comparable E4 levels but with fully sensitive AChE. 1200Q with the same E4 level but with homozygously insensitive AChE, had a resistance factor of > 50 over the same clone (Table 2).

TABLE 2. Sensitivity of aphid clones to pirimicarb (aphid dip)

Clone	E4 level	AChE	LC_{50} ppm	95% CL	Slope	RF
US1L	S	S/S	23	16-32	1.26	1
T1V	R_2	S/S	200	140-270	1.42	9
1051A	R_2	S/I	2800	1920-4810	1.87	120
1200Q	R_2	I/I	Not calculable *			>500

* 50% kill impossible due to limiting solubility of pirimicarb

TABLE 3. Sensitivity of aphid clones to triazamate (systemic)

Clone	E4 level	AChE	LC_{50} ppm	Limits	Slope	RF
US1L	S	S/S	0.4	0.16-101	1.28	1
T1V	R_2	S/S	2.5	0.61-601	1.17	6
1051A	R_2	S/I	72	32-180	0.97	160
1200Q	R_2	I/I	330	140-1180	0.84	730

Similarly, 1051A had triazamate resistance of 25-fold over a comparable E4 clone, whilst the 1200Q gave a factor of > 100 (Table 3). This dramatic effect of the modified AChE, whether homozygous or heterozygous, means that in the presence of this mechanism, continued use of compounds to which it confers resistance will rapidly intensify the porblem. Although the mechanism appears to be rare in *Myzus* populations at present, it is important to supplement esterase-based monitoring for resistance with the rapid diagnostic tests developed (Moores *et al.*, 1988) to identify the presence of insensitive AChE so that appropriate control tactics can be adopted.

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CHALLENGES IN PRODUCING RESISTANCE MANAGEMENT STRATEGIES FOR *MYZUS PERSICAE*

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ABSTRACT

Insecticide resistance is widespread in *Myzus persicae*, causing control problems in annual crops and its primary host, peach. Several aspects of the biology and nature of resistance in this species have combined to make the production of resistance management guidelines a challenge. These include a single predominant resistance mechanism conferring cross-resistance to all major classes of insecticide, application of insecticides to both summer and winter hosts, and crop protection measures frequently dictated by other pests occurring simultaneously with *M. persicae*.

INTRODUCTION

The IRAC (Insecticides Resistance Action Committee) Fruit Crops Working Group produced guidelines for the resistance management of spider mites in top fruit and citrus (Leonard & Wege, 1994). The guidelines are purposely simple and pragmatic to facilitate their adaptation to specific situations. Their successful integration into a Belgian IPM system (Sterk & Highwood, 1992) demonstrates the validity of this approach. The present paper discusses the problems encountered in attempting to devise a similar set of guidelines for managing insecticide resistance in *Myzus persicae*, and aims to initiate dialogue on this challenging problem.

INSECT CONTROL ON PEACH

Where the holocyclic life cycle predominates, *M. persicae* is a principal pest of the primary host, peach and nectarine (*Prunus persica*) in all major growing areas. Fundatrices emerge from eggs in the spring and give rise to apterous parthenogenetic females, of which there may be several generations. These can cause serious damage by feeding on newly opened leaves, or distortion or premature fall of fruit by feeding on fruits and flowers. In mid summer, alates are produced and migration to secondary host plants occurs. Alates return to the primary host in the late summer where they give rise to sexual forms which, following sexual reproduction, lay eggs in crevices in the bark. A proportion of the population may overwinter on peach as parthenogenetic females (Cravedi & Cervato, 1991).

As a consequence of the pest status of *M. persicae* in most major peach growing areas, a large proportion of regional populations may be treated with 1-5 foliar applications of insecticide and are thus subject to intense selection pressure. The first application is made pre-flowering or immediately post-flowering, although the exact timing varies regionally and may be made according to calendar (e.g. Japan), crop growth stage (e.g. Spain), the appearance of

other, locally more significant pests (e.g. plum cuculio in USA), the first sign of aphids or on carefully determined thresholds (e.g. Italy and France).

Spray thresholds are typically set at low population levels, for example, the pre-flowering threshold for nectarines in the Emelia Romagna region of Italy is 1% of shoots infested. Foliage is minimal at this time, and effective spray coverage can be achieved. At this point in the life cycle of holocyclic *M. persicae*, there is maximum diversity in levels of resistance within the population. Treatment at this time and under these conditions contributes to effective control and helps prevent the build up of large populations of (resistant) individuals.

Choice of insecticides used for aphid control is often influenced by the local significance of other early season pests, including thrips (*Frankliniella occidentalis*, *Thrips meridionalis*) scale (*Quadraspidiotus perniciosus*), pentatomid and mirid bugs (e.g. *Lygus lineolaris*, *Acrosternum hilare*), Coleoptera (*Conotrachelus nemuphar*), and Lepidoptera (*Grapholitha molesta*, *Adoxophyes orana*, *Synanthedon hector*). The necessity for broad spectrum control limits the choice of products available to the grower and restricts the options available for resistance management, especially where product alternation or rotation strategies are favoured. Alternatively, additional treatments are necessary to control these or other later occurring pests, further exposing the residual aphid population to insecticides.

MYZUS RESISTANCE IN PEACH

One resistance mechanism, that of elevated levels of carboxylesterase (E4) enzymes which can neutralise insecticides through hydrolysis or sequestration, appears to predominate in *M. persicae* (Devonshire & Moores, 1982). The mechanism confers cross-resistance to carbamates, organophosphates (OP's) and pyrethroids (Sawicki & Rice, 1978). Individual aphids may be broadly classified as susceptible, moderately resistant (R1), very resistant (R2) or extremely resistant (R3) according to the quantity of E4 present.

Resistance to one or more classes of insecticide has been reported for *M. persicae* in most of the major peach growing areas (Voss, 1987). Samples of *M. persicae* from Australian peach have been shown to consist predominantly of R2 individuals (Herron *et al.*, 1993) whereas samples taken in Greece, Spain, Portugal, France and Italy have been found to consist predominantly of R3 aphids (Table 1). Against such populations, little, if any control is obtained with many OP insecticides and the efficacy of carbamate insecticides is often reduced. Pyrethroids surprisingly remain effective in many areas, even against populations consisting of predominantly R3 strains, which show strong pyrethroid resistance in bioassays. This might be explained by the initial, transient effects of the compounds (hyperactivity and ataxia) causing aphids to fall from the foliage.

Specific resistance mechanisms

Specific resistance mechanisms have been identified in other species of aphid, for example pirimicarb- and OP-insensitive acetylcholinesterases have been identified in *Aphis gossypii* (Silver, 1984; Sun *et al.*, 1987). In certain peach growing localities where the extensive and sole use of pirimicarb has occurred over many years, *M. persicae* with acetylcholinesterase specifically insensitive to pirimicarb and triazamate have been discovered.

This mechanism was found to occur both in conjunction with, and separately from elevated levels of esterase (Moores *et al.*, 1994). As an example of a specific, altered site resistance mechanism in *M. persicae*, this emphasises the consequences of heavy use of a single product, which may initially be highly effective against current resistant strains. The lesson is particularly relevant to recently launched novel aphicides, which are largely unaffected by the E4 resistance mechanism.

TABLE 1. Percentages of resistant and susceptible *Myzus persicae* sampled from primary and secondary host plants.

Country	Year	Crop	S	R1	R2	R3	n*
Germany	1992	potato	2	48	49	1	400
Germany	1990	sugar beet	18	59	23	0	42
Denmark	1991	beet clamps	1	96	3	0	937
France	1991	potato	6	35	51	8	168
Spain	1991	nectarine	0	4	28	68	84
Portugal	1991	peach	1	6	22	71	365
Italy	1991	peach	0	0	8	92	168
Greece	1990	tobacco	0	0	1	99	168
Greece	1990	peach	0	0	47	53	84
Argentina	1993	peach	0	42	54	4	79

* n=number of aphids in sample.
Samples provided by Zeneca. Esterase levels determined by A.L. Devonshire at Rothamsted.

INSECT CONTROL ON SECONDARY HOSTS

Crops which serve as secondary host plants for *M. persicae* are frequently found in close proximity to peach or nectarine orchards, often cultivated by the same grower. These are typically vegetables (e.g. France, Italy, Spain, South Africa, Turkey), potatoes (e.g. Argentina, South Africa) and tobacco (e.g. Greece and Turkey). Other secondary host plants of which *M. persicae* is an important pest include sugar beet and oilseed rape. Occasionally populations may build up sufficiently to cause physical damage, however, the species has a tendency to disperse, and economic damage most frequently results from the transmission of plant viruses. In many regions of Northern Europe, the primary host is uncommon and holocyclic forms appear rare (Broadbent & Heathcote, 1955). Aphids overwinter parthenogenetically on secondary host plants such as autumn sown brassicas, oilseed rape, beet clamps, and weeds.

Where virus control is the primary objective, sprays of pyrethroids or pyrethroid mixtures are often favoured over other chemical classes for their effects on virus transmission (Gibson, 1983). Spraying to carefully determined thresholds is common for virus control in potato and sugar beet (e.g. Dunning, 1986; Bacon *et al.*, 1978), however, there is a tendency amongst growers to make insurance sprays which increases selection pressure.

Soil and seed treatments for the control of *M. persicae* or other aphids are becoming

increasingly popular in a number of annual crops. The consequences of this in terms of resistance are not yet fully understood. Situations will increasingly arise where aphids move from one treated crop to another without exposure to different chemistry. Where resistance exists, soil applied treatments have been shown to contribute to its development in aphids (Hardee & Ainsworth, 1993), however, they may generate fewer problems than the alternative of foliar insecticide applications principally due to preservation of beneficials.

Insecticide applications are frequently required to control other insect pests which occur simultaneously with aphids, for example *Leptinotarsa decemlineata* and *Pththorimaea operculella* in potatoes, *Ostrinia nubilalis* and other Lepidoptera in pepper, and *Heliothis sp.* in tobacco. The necessity to control these insects leads to increased selection pressure for *M. persicae* resulting from additional chemical applications or the use of compounds which may be less favourable in terms of selecting for resistance.

Rapid parthenogenetic multiplication on secondary hosts greatly multiplies the genotype of resistant individuals. Applications to the secondary host may therefore strongly influence the genotype entering the sexual phase and therefore the resistance status of the fundatrices of the following season. Resistance management practices implemented on peach may be compromised if complementary efforts are not made on the secondary hosts in the vicinity. Treatment of summer and winter hosts also occurs in areas where *M. persicae* overwinters parthenogenetically; for example winter oil seed rape, a preferred winter host of *M. persicae*, is increasingly treated in the late autumn with pyrethroids in the UK for the control of aphid transmitted virus, cabbage stem flea beetle and flea beetles.

MYZUS RESISTANCE IN SECONDARY HOSTS

Control problems can occur in populations that consist of a large proportion of R2 or R3 aphids. The distribution of these strains has been extensively studied in the UK (Furk *et al.*, 1990; Smith & Furk, 1989; ffrench-Constant & Devonshire, 1988; Sawicki *et al.*, 1978). Over the last 20 years, the proportion of susceptible aphids has remained low in the UK and the proportion of the very resistant R2 types has gradually increased, with a corresponding increase in control failures in sugar beet and potatoes. Similar trends have been observed in other European countries such as Northern France, Belgium and Denmark. The appearance of significant proportions of R3 strains in these areas has generally been slow, possibly due to the inferior overwintering capability of these strains (Hockland *et al.*, 1992). Although insecticide resistance in *M. persicae* in secondary crops is widely documented, few workers have fully characterised aphid populations in terms of esterase levels. High proportions of R2 aphids have been reported in populations from New Zealand potatoes (Cameron & Walker, 1989) and very high levels of R3's occur in Greek tobacco (Table 2).

PRESENT RESISTANCE MANAGEMENT

A variety of measures is used to manage the effects of resistance in *M. persicae* in peaches. These include increasing rates, tank mixtures and alternation of chemical types. The prime motive behind these measures is maintaining control in the short term rather than extending the useful life of the currently available compounds by preventing the build up of resistant strains. There is little evidence of rationally based resistance management in

secondary hosts based on alternation or mixing of different products. Exceptions may be found in the UK, where recommendations have been made to reduce the selection of resistant strains in sugar beet by limiting pyrethroid-OP mixtures to the first application (Dewar *et al.*, 1992), and by the use of carbamate compounds such as pirimicarb and aldicarb, which select less strongly for resistant strains. The lack of technically based resistance management guidelines for product use is probably a reflection of the difficulty in formulating meaningful guidelines.

POSSIBLE TOOLS FOR RESISTANCE MANAGEMENT

There are considerable differences in the degree to which OP's, carbamates, pyrethroids and newer compounds of different chemistry (and individual compounds within those classes) are affected by E4 in *M. persicae* (Devonshire & Moores, 1982). The recently released novel compounds (imidacloprid, diafenthiuron and pymetrozine) are largely unaffected. Resistance management guidelines could be based around resistance factors (RF) which are a measure of inactivation by E4. RF values are calculated from the ratio of LC_{50} values between resistant and susceptible strains. Resistance factors between R2 and R1 *M. persicae* in contact bioassays for a range of insecticides are presented in table 2. These data, generated by Zeneca, are broadly consistent with those of Sawicki & Rice (1978) and Sawicki *et al.* (1978).

TABLE 2. Average resistance factors (RF) at LD_{50} for R2 vs. R1 *Myzus persicae*.

Compound	Chemical class	RF (LC_{50} R2/LC_{50} R1)	n
imidacloprid	chloronicotinyl	1	1
propoxur	methylcarbamate	1	2
triazamate	carbamyl triazole	3	2
acephate	methylphosphate	4	1
pirimicarb	dimethylcarbamate	8	5
diazinon	diethylphosphate	14	1
tefluthrin	ester pyrethroid	15	5
endosulfan	cyclodiene	20	1
heptenophos	dimethylphosphate	21	1
flufenprox	ether pyrethroid	22	5
fenvalerate	ester pyrethroid	33	5
permethrin	ester pyrethroid	40	5
cypermethrin	ester pyrethroid	59	5

*n= number of tests.

The use of this index as a tool for resistance management guidelines is supported by field data generated in the UK by Dewar *et al* (1992), Smith & Devonshire (1990) and ffrench-Constant *et al.* (1987): These data demonstrate (albeit for a limited range of products) that compounds with higher RF values, such as pyrethroids and some OP's, select for resistant

strains more rapidly than those less affected by E4 esterases such as carbamates. No such work has yet been completed for *M. persicae* on the primary host.

RF values derived from a single set of clones characterised for varying levels of esterase could provide a reliable basis for resistance management guidelines. Amongst numerous field samples bioassayed using a variety of techniques (Choi & Kim, 1986; Attia & Hamilton, 1978; Cameron & Walker, 1989; Cloquemin *et al.*, 1990 and Herron *et al.*, 1993) there was no strong evidence of significant departure from the trends in RF values suggested by Sawicki & Rice (1978) and rationalised biochemically by Devonshire & Moores (1982). Absolute values do differ and are dependent on bioassay technique (Sawicki & Rice, 1978). This is particularly relevant for pyrethroids, the repellant properties of which can lead to avoidance of treated surfaces, resulting in artificially high RF values. The presence of specific resistance mechanisms in *M. persicae*, arguably compromises the use of RF's based on single strains of aphids. However, these strains appear strictly localised and the E4 mechanism can, at present, be considered valid for the majority of situations.

Further work to establish the relationship between laboratory generated RF values and field efficacy for a wider range of compounds and crops is required in order to determine the practical significance of this approach. These studies should also incorporate factors such as the persistence of foliar applied insecticides, selectivity to beneficials, and possible physiological effects such as the stimulated nymph production which can be associated with some compounds, all of which can affect the rate at which resistant *M. persicae* are selected or the rate at which resistant populations become established (ffrench-Constant *et al.*, 1987)

IRAC GUIDELINES FOR RESISTANCE MANAGEMENT.

At present, the IRAC Fruit Crops and Field Crops Working Groups are unable to define a general set of practical guidelines for the resistance management of *M. persicae* in the manner proposed for spider mites (Leonard & Wege, 1994). However, a number of basic instructions can be proposed:

1. Use compounds at the manufacturers' recommended rates and timings. Under no circumstances should application rates be increased over and above label recommendations.

2. Ensure effective coverage of the infested part of the crop.

3. Tank mixtures should employ the full label rates of both compounds.

4. Compounds should be used in such a way that impact on beneficial populations is minimised.

5. Monitoring should be used to detect the early signs of resistance*.

6. Where specific resistance mechanisms are identified, further exposure of the population to that active ingredient should be avoided.

*Resistance monitoring methods for *M. persicae* are described by IRAC (1990) and, in addition, biochemical and ELISA assays for E4 activity or quantity have been developed (e.g. Devonshire & Moores, 1984; Devonshire, 1975).

IRAC welcomes further dialogue and ideas from research workers, advisors, and growers on resistance management guidelines and their implementation.

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INSECTICIDE RESISTANCE ACTION COMMITTEE (IRAC) FRUIT CROPS SPIDER MITE RESISTANCE MANAGEMENT GUIDELINES 1994

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ABSTRACT

A principle objective of the Insecticide Resistance Action Committee (IRAC) is to delay or prevent the onset of insecticide resistance through the development of resistance management guidelines and their subsequent implementation as part of IPM or other production schemes. In line with this objective, IRAC Fruit Crops Working Group (FCWG) produced guidelines for spider mite resistance management in 1988. The present paper represents the second revision of these guidelines incorporating both newly available acaricide compounds and an extension of the crops covered by the guidelines.

INTRODUCTION

IRAC's guidelines for resistance management of spider mites in top fruit first published in 1988 (Lemon, 1988). An IRAC sponsored research programme (Dennehy & Wentworth, 1991) and further experiences within the individual companies represented on the IRAC Fruit Crops Working Group (FCWG) led to a revision of these guidelines (Leonard, 1992).

Five important new acaricides have subsequently appeared on the market, namely diafenthiuron, fenazaquin, pyridaben, tebufenpyrad and fenpyroximate. There is strong evidence to suggest that the latter four of these share a common mode of action acting on the rotenone site in the mitochondrial respiration electron transport chain (Hollingworth *et al.*, 1992, 1993; Motoba *et al.*, 1992; Anon, 1993a). Bioassays undertaken in Japan on *Panonychus citri* have indicated the presence of strains with reduced susceptibility to respiration inhibitor acaricides in certain locations (Anon, 1993b; Anon, 1994). Further tests on one of these strains by a company represented on the FCWG suggested cross-resistance between the four compounds. For this reason, the four compounds have been placed in one group in the compound groupings. There are no data available to suggest that strains of mite resistant to diafenthiuron have yet developed.

In accordance with developments in mite control practices in top fruit, the guidelines now recognise that reduced rates of application can be adopted, but only as part of established Integrated Pest Management (IPM) schemes in which the effects of acaricide treatments are augmented with the use of beneficial organisms. This can only be undertaken under the guidance of, and at the responsibility of, the local advisory services.

The scope of the guidelines has also been broadened to include citrus. The revised 1994 compound groupings and use guidelines are shown in full below:

COMPOUND GROUPINGS

The following list of compounds was compiled on the grounds that cross-resistance is likely to exist between compounds in the same group.

Group A	Organotins
Group B	Clofentezine, hexythiazox, flufenoxuron and flucycloxuron
Group C	Bridged diphenyl compounds (bromopropylate and dicofol)
Group D	Pyrethroids
Group E	Tetradifon
Group F	Amitraz
Group G	Propargite
Group H	Quinomethionate
Group I	Benzoximate
Group J	Dinobuton
Group K	Abamectin
Group L	Organophosphates
Group M	Formetanate
Group N	Diafenthiuron
Group O	Fenazaquin, tebufenpyrad, fenpyroximate and pyridaben

USE GUIDELINES

The following guidelines are recommended for use with the above product groupings:

1. Not more than one compound from any one group should be applied to the same crop in the same season*.

2. Any one compound should be used only once per season on any one crop.

3. Mixtures of acaricides from different groups may be used but use of mixtures of products from the same group is not recommended.

4. Compounds should be used in such a way that detrimental effects on predatory insects and mites are minimised.

5. Use compounds only at manufacturer's recommended rates and timings**.

6. Monitoring should be conducted to detect early signs of resistance***.

7. Oils may be used in the spring to reduce mite pressure whilst contributing to scale control (eg. *Aonidiella aurantii* in citrus).

* Where more than one mite species needs to be controlled, **compounds from the same group should not be re-applied.**

** **Manufacturers recommended dosage rates which reliably control pests under normal conditions should be used as recommended on the label.**

Under certain circumstances, WITHIN the recommendations of local advisory services, rates of use below those recommended by manufacturers may be used in combination with established IPM systems. This procedure may reduce selection pressure and hence, resistance development. Under these circumstances the chemical treatment is supplemented with the use of introduced beneficials, or methods used to encourage natural enemies. Careful monitoring of pest numbers is necessary, as is effective use of damage thresholds (these have not yet been developed for *Panonychus citri*. Further research in this area would be most useful).

Such recommendations must be acceptable under local law, and remain the responsibility of the local advisory service. **Under no circumstances should doses above the manufacturer's recommendations be used.**

*** Resistance monitoring methods have been proposed for *Tetranychus urticae* and *P. ulmi* adults and eggs (OEPP/EPPO, 1990).

IMPLEMENTATION

The IRAC spider mite resistance guidelines are intended to provide a technically sound foundation to locally based IPM or resistance management programmes, rather than a strategy per se. Sterk & Highwood (1992) and Sterk (1992) describe an example where these guidelines were specifically adapted and successfully incorporated into an IPM programme in apple orchards in Belgium under the guidance of the Gorsem Research Institute. There is every chance that under systems such as this, resistance management will be successful. However, it is recognised that growers and advisors do not always facilitate this process by using products, or recommending their use, in a manner which is inconsistent with the principles of resistance (Leonard & Perrin, 1994). In order to further assist the implementation of guidelines, IRAC intends to raise the profile of resistance management guidelines amongst the user community by targeted publication in formats accessible to growers and advisors. In addition, the FCWG are striving to include IRAC guidelines, or recommendations for uses which are entirely consistent with IRAC guidelines, on the acaricide product labels.

FURTHER DEVELOPMENTS

IRAC are currently sponsoring a study at Rothamsted Experimental Station to evaluate a simple, leafless bioassay for monitoring the susceptibility of *P. ulmi* to the four new respiration inhibitor acaricides. If the method proves suitable, it will provide a valuable tool for the successful resistance management of these compounds, and a useful source of baseline data for future studies investigating changes in susceptibility to these compounds.

Further work is required to investigate the patterns of cross-resistance amongst strains of mite resistant to the respiration inhibitor acaricides. There is evidence to suggest that cross resistance extends to acaricides beyond these four compounds (Richter & Otto, 1992). If this is confirmed, further revision of the spider mite guidelines may be necessary.

Guidelines and compound groupings will continue to be reviewed as further data on factors affecting selection of acaricide resistance become available and as new products enter the market. Comments and ideas from academia, growers and advisors are welcomed by the FCWG to assist in this process.

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INSECTICIDE RESISTANCE IN BEMISIA TABACI FROM PAKISTAN

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ABSTRACT

Cotton production in Pakistan has declined markedly over the last two seasons. This was as a consequence of several factors, including unprecedented infestations by cotton leaf curl virus (CLCV) and its vector the cotton whitefly (Bemisia tabaci). Research at Rothamsted and in Pakistan has disclosed moderate to very strong resistance in B. tabaci to all of the commonly-used insecticides, supporting frequent claims that these chemicals are losing their effectiveness under field conditions. These findings reinforce the urgency of identifying and implementing sustainable pest management practices for the cotton cropping system.

BACKGROUND

The tobacco or cotton whitefly, Bemisia tabaci, is now a cosmopolitan pest attacking a wide range of arable, horticultural and ornamental crops. On cotton it achieves significance as a vector of plant geminiviruses and as a cause of 'stickiness' due to honeydew production, which impedes harvesting and ginning, and impairs fibre quality. In one or both of these capacities, B. tabaci has recently erupted to plague proportions on cotton in Central America, the USA, North Africa, the Middle East and the Indian Subcontinent. In some of these regions it is now considered the most economically-damaging and intractable insect pest.

Problems caused by B. tabaci are well-exemplified by recent events in Pakistan, whose cotton industry sustains the livelihood of over 12 million workers and their dependents (Ingram et al., 1989). Excluding a dramatic crop failure in 1983, due to severe floods and exceptional pest pressures, production increased rapidly from 1980 to reach a peak of 2.1 million tonnes in 1991 (Fig. 1), making Pakistan the world's fourth largest producer behind China, the USA and the former Soviet Union (International Cotton Advisory Committee, 1993). These improvements, attributable primarily to increases in yield rather than total cotton area (c. 2.5 million hectares per annum), reflect the development of new heat-tolerant varieties, improved agronomic practices, and intensified pest control with chemical pesticides. Over the last two years, however, production and yield have declined rapidly, to reach, in 1993, only 60% of the 1991 peak (Fig. 1). This decline represents a major crisis for Pakistan which is dependent on cotton for over 60-70% of its hard currency export earnings and over 50% of its edible oil requirements.

Several events have contributed to the yield losses, including extensive flooding in 1992 that hampered normal crop husbandry and pest management practices. However, significant damage was caused directly by B.tabaci through feeding and honeydew production, and indirectly through its transmission of cotton leaf-curl virus (CLCV). CLCV has been

indirectly through its transmission of cotton leaf-curl virus (CLCV). CLCV has been recognised in Pakistan since the late 1960s, but was relatively trivial prior to a dramatic expansion in its range and severity that commenced in the late 1980s and led to several hundred thousand acres of cotton being affected to a varying extent in the 1992 and 1993 seasons.

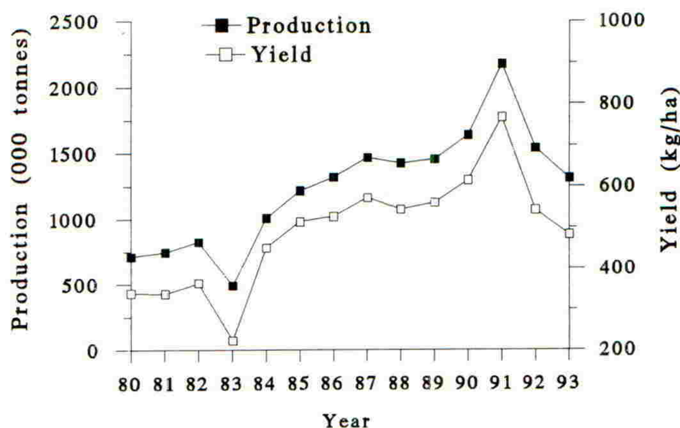

FIGURE 1. Production and yield of cotton in Pakistan for the period 1980-93 (Source. International Cotton Advisory Committee, 1993)

CONTROL OF COTTON PESTS

As in many countries, insecticides are now the mainstay for controlling B. tabaci and other cotton pests including thrips, jassids and a complex of pink (Pectinophora gossypiella), spotted and spiny (Earias spp.) and American (Helicoverpa armigera) bollworms. In recent years, emphasis has been on inexpensive organophosphorus (OP) compounds such as monocrotophos and methamidophos against early season sucking pests, followed by calendar spray of pyrethroids or, more commonly, pyrethroid/OP mixtures against the bollworm complex and/or late season outbreaks of sucking pests. Both the area and total volume of these products has increased steadily since 1980 (Ingram et al., 1989), with over 50% of farmers applying four or more sprays per season by 1990 (Z. Ahmad pers. comm. 1992). Although not excessive by international standards, this trend has prompted concerns over the likely selection of insecticide resistance, particularly in those species (including B. tabaci and H. armigera) that have developed resistance in other cotton-producing countries.

RESISTANCE PROJECTS INVOLVING B. TABACI

Three projects are currently addressing these concerns by investigating the occurrence and extent of resistance in cotton pests in Pakistan. The longest-standing of these, involving both B. tabaci and H. armigera, is based at the Central Cotton Research Institute (CCRI) in Multan and forms the nucleus of resistance work in the public sector in Pakistan. A second large-scale monitoring programme was established in 1992 at the University of

Faisalabad under sponsorship from the Insectide Resistance Action Committee (IRAC) Cotton Pakistan (ICP), a consortium of the major national and international agrochemical producers and distributors. This also focusses on B. tabaci and H. armigera, and uses the same bioassay methods as at CCRI. The third project was initiated in 1993 at Rothamsted Experimental Station with support from the UK Natural Resources Institute. This deals only with B. tabaci, complementing other studies by concentrating on fewer strains to examine cross-resistance patterns in more detail, developing new bioassay methodology, and providing initial data on resistance mechanisms. In this paper we present preliminary information on resistance and its implications in two strains collected from different hosts and at different times of the year.

INSECTS AND INSECTICIDES

Resistance profiles are reported for two field strains of B. tabaci, termed PAK-1 and PAK-6 respectively. The former was collected from cotton at Rahim Yar Khan, near the southern border of Punjab province in November 1992, towards the end of the cotton cropping season. The latter was collected in August 1993 from a field of brinjal (aubergine, *Solanum melongena*) to the south of Faisalabad, c. 200km north of the major and most-intensively sprayed cotton-growing regions. After collection, both strains were maintained at Rothamsted without insecticide selection on cotton plants (Gossypium hirsutum var. Deltapine 16) in a 16h photoperiod at 26°C. To provide adequate numbers of B. tabaci of known age, each rearing cage was kept for just one generation and adults aged between 2 and 8 days old were used for bioassays. The reference strain SUD-S has been maintained at Rothamsted since 1985 and exhibits baseline susceptibility to all chemicals tested.

Bioassay are reported here for eight of the insecticides or insecticide combinations currently in widest use on Pakistani cotton. Formulations obtained from their respective manufacturers were: cypermethrin (Polytrin, 20% EC; Ciba-Geigy); cyfluthrin (Baythroid, 5% EC; Bayer); profenofos (Curacron, 50% EC; Ciba-Geigy); monocrotophos (Nuvacron, 40% SCW; Ciba-Geigy); methamidophos (Tamaron, 60% SL; Bayer); a commercial mixture of profenofos and cypermethrin (Polytrin C, 40% profenofos and 4% cypermethrin EC; Ciba-Geigy); a commercial mixture of methamidophos and cyfluthrin (Baythroid TM, 50% methamidophos and 2.5% cyfluthrin EC; Bayer); and endosulfan (Thiodan, 35% EC; Hoechst).

LEAF-DIP BIOASSAYS

For leaf-dip bioassays, adult female whiteflies were confined to a cotton leaf disc (37mm diameter) laid on an agar bed and held inside a ventilated plastic Petridish (38mm diameter, 20mm high). Leaf discs were dipped into insecticide diluted to the required concentration with a 0.01% aqueous solution of the non-ionic wetter 'Agral' (Zeneca Agrochemicals) to ensure adequate wetting at very low insecticide concentrations. Controls were treated in the same manner except that the leaf discs were dipped in the diluent only. All tests were maintained at 25°C and final mortality was scored after 48h. Bioassays against field strains used three replicates of 20-30 insects at a minimum of five concentrations. Results were subjected to probit analysis using the software package POLO-PC (LeOra Software, Menlo Park, California). Resistance Factors (RFs) were calculated at LC_{50} relative to the response

of the SUD-S strain.

TABLE 1. LC_{50}s (mg/l), (95% confidence limits in parentheses) and resistance factors for the PAK-1 and PAK-6 strains in adult leaf-dip bioassays.

	SUD-S	PAK-1			PAK-6		
Insecticide	LC_{50}	LC_{50}	limits	*RF*	LC_{50}	limits	*RF*
cypermethrin	3.6	200	(150 - 270)	*56*	>1000		*>300*
profenofos	6.1	340	(230 - 440)	*56*	120	(100 -140)	*20*
cyp. & prof.	2.4	86	(62 - 110)	*36*	100	(60 - 140)	*42*
cyfluthrin	0.52	43	(26 - 60)	*83*	>1000		*>2000*
methamidaphos	14	960	(650 - 1200)	*69*	990	(670 - 1200)	*71*
cyf. & metham.	6.9	500	(420 - 570)	*72*	800	(690 - 920)	*116*
monocrotophos	14	560	(440 - 700)	*40*	120	(80 - 160)	*9*
endosulfan	0.51	6.8	(5.0 - 8.6)	*13*	11	(8.8 - 14)	*22*

There was moderate to very strong resistance to all insecticides tested, extending to pyrethroid/OP mixtures as well as their individual constituents (Table 1). RFs for OPs and both mixtures were broadly comparable and ranked in a similar manner for the two populations. Figures for cypermethrin and cyfluthrin differed substantially between strains, with PAK-6 exhibiting virtual immunity to these chemicals in leaf-dip tests. This finding is initially surprisingly, given that pyrethroids are not specifically recommended against B. tabaci on Pakistan cotton. However, current reliance on pyrethroid/OP mixtures for broad-spectrum pest control must result in extensive whitefly exposure to pyrethroids, and no doubt accounts for resistance levels equal to or exceeding those in many other countries (Dittrich et al., 1990; M. Cahill et al., unpublished data). Confirmation of resistance to endosulfan is also of major concern given the likely increasing reliance on this compound as an alternative to OPs during future cropping seasons (Z. Ahmad, pers. comm. 1994).

IMPLICATIONS FOR FIELD CONTROL

Implications of these results for the efficacy of currently-used insecticides have yet to be assessed directly in the field, but have been investigated extensively at Rothamsted under simulated field exposure conditions (M. Cahill et al., unpublished data). Techniques for the maintenance, treatment and monitoring of B. tabaci in 'field simulator' chambers have been described previously (Rowland et al., 1990). For present purposes, c. 400 adults of either SUD-S or a recently-collected Pakistan strain were released onto eight cotton plants within each cage (1.7m x 1.2m x 1m), sprayed with recommended field rates of formulated cypermethrin (65g a.i./ha) or methamidophos (500g a.i./ha), and their numbers monitored at 1-3 day intervals thereafter. Representative results for two chemicals, cypermethrin and methamidophos, are shown in Figure 2.

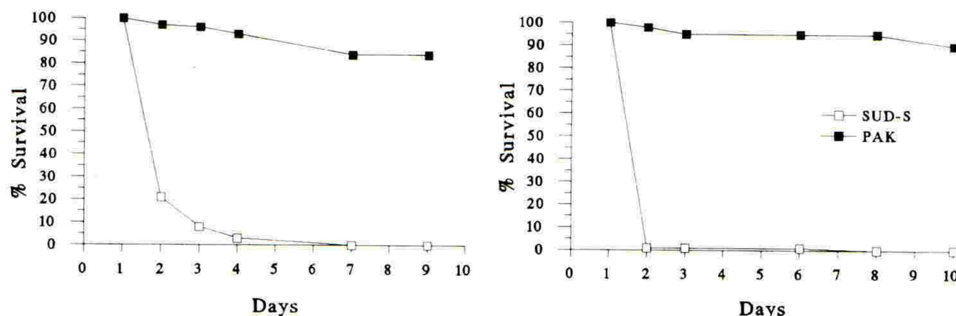

FIGURE 2. Survival of susceptible and Pakistani whiteflies treated in field simulators with recommended field rates of cypermethrin (left) and methamidophos (right).

Both chemicals were highly effective at controlling SUD-S adults. In contrast, adults from Pakistan were virtually unaffected in both cases, showing only the very protracted decline in numbers consistent with natural mortality in untreated populations.

PROSPECTS FOR RESISTANCE MANAGEMENT

Results accumulating from all three monitoring projects are consistent and have alarming repercussions for combating whitefly and virus problems on Pakistani cotton. They not only show resistance to be well-established in B. tabaci, but also back up numerous anecdotal accounts of insecticides exerting little or no control of whitefly infestations. Resistance is clearly also geographically widespread, extending well beyond the major cotton belt and to vegetable hosts acting as important reservoirs of B. tabaci both within and outside the cotton season.

Currently, any attempt to manage insecticide resistance in Pakistan by rationalising and reducing insecticide inputs is greatly complicated by the severity of the CLCV epidemic. This has already inflicted heavy losses on growers, and has understandably become the most feared threat of all to the cotton industry. Until the virus problem abates, or more tolerant cultivars become widely available, the intuitive but ineffective approach to virus prevention of spraying the vector 'on sight' and with repeated applications of insecticide seems destined to continue. This, coupled with the often instinctive response to control failures of raising application rates or respraying even more frequently runs a definite risk of consolidating and broadening resistance problems still further.

Fortunately there are other factors that could favour an effective long-term solution. Firstly, the pesticide industry is still far more structured in Pakistan than in many other developing countries. Over 90% of insecticides are sold by less than a dozen manufacturers or distributors, whose representation on ICP implies a commitment to IRAC's publicly-stated goal of promoting and implementing resistance management worldwide (eg. Leonard &

resistance, careful stewardship will be vital to sustain their effectiveness. Thirdly, developments over the last two years have amply highlighted the urgency with which Pakistani authorities must implement and exploit research in all areas relevant to combating resistance. Close scrutiny and coordination of both Government- and Aid-funded projects on cotton pest management must be seen as a prerequisite for the continued profitability of a crop on which the economic development of Pakistan is so critically dependent.

ACKNOWLEDGEMENTS

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INSECTICIDE RESISTANCE IN *HELICOVERPA ARMIGERA* IN INDIA: RECENT DEVELOPMENTS

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ABSTRACT

Seasonal changes in insecticide resistance in the cotton bollworm, *Helicoverpa armigera* (Hübner) were monitored at six widely spaced locations in India during 1993/94 using a discriminating dose bioassay technique. Insecticide / synergist combinations were used to elucidate resistance mechanisms. The status of resistance to pyrethroids, endosulfan and organophosphates is described and correlated with local farmer insecticide use strategies in the different regions and the role of migration in the spread of resistance beyond high input farming areas.

INTRODUCTION

Severe pyrethroid resistance in the cotton bollworm, *Helicoverpa armigera* (Hübner) was first recorded from cotton in eastern Andhra Pradesh, India in 1987 (Dhingra *et al.*, 1988; McCaffery *et al.*, 1989). Associated with high population pressure, field control failures and a marked decline in lint yields (from 430 to 170 kg/ha) resulted. More recently, bioassay of field collected material using discriminating doses of synthetic pyrethroids (SPs), organophosphates (OPs) and endosulfan, and various synergist combinations at monitoring centres established at six locations in India has enabled its spread, and local changes in resistance intensity and their mechanisms to be followed closely for the first time (Armes *et al.*, in press). Correlation of resistance with records of local insecticide use patterns has generated much useful information relevant to the development of management strategies, some of which are currently being tested.

MATERIALS AND METHODS

Insecticide resistance monitoring laboratories have been established in six widely spaced pulse and cotton growing locations in India, *viz.*: Andhra Pradesh Agricultural University (APAU), Guntur; Central Institute for Cotton Research (CICR), laboratories at Coimbatore, Nagpur and Sirsa; International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad; Tamil Nadu Agricultural University (TNAU) campuses at Coimbatore and Madurai, (Fig. 1).

H. armigera eggs were collected from host plants in farmers' fields over as much of the 1993/94 cropping season as possible at the six locations. Typically the range of major host plants sampled over the season comprised: wild host plants, tomato, sorghum, cotton, sunflower, pigeonpea, chickpea and groundnut. Several thousand eggs were collected weekly from each region and transferred individually to 7.5ml cells of 12-well tissue culture plates containing a chickpea artificial diet. Once the larvae reached the 30-40 mg weight range they were randomly assigned to 6 discriminating dose screens *viz.*: cypermethrin 0.1 μg/μl, fenvalerate 0.2 μg/μl, endosulfan 10.0 μg/μl, quinalphos 0.75 μg/μl (approximations to LD_{99} values for homozygous insecticide susceptible *H. armigera* strains); cypermethrin 1.0 μg/μl (introduced as a "twin" discriminating dose because of the high survival at the 0.1 μg/μl dose), cypermethrin 0.1 μg + piperonyl butoxide (Pbo) 50 μg/μl (to determine the extent of Pbo suppressible pyrethroid resistance) and at the Central region only, cypermethrin 0.1 μg + profenofos 0.1 μg/μl (to determine the extent of profenofos suppressible

pyrethroid resistance). For each location, each week at least 100 (ideally 120-250) larvae were treated topically with 1.0 µl of each discriminating dose. Rearing and insecticide assays were conducted in rooms maintained at a constant temperature of 25 ± 2 °C, under natural photoperiod (approx. 12:12 h light:dark).

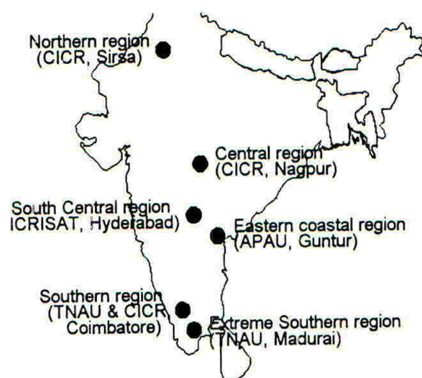

FIGURE 1. Location of *H. armigera* insecticide resistance monitoring laboratories in India.

RESULTS

In view of the large amount of data, it is not possible to present all the results in this paper. However, seasonal SP, endosulfan and OP insecticide resistance data for indicator regions: Haryana, Maharashtra, coastal Andhra Pradesh and Tamil Nadu are presented graphically in Figures 2-4. Data for the South Central region are presented elsewhere (Armes *et al.*, in press), but summarised briefly along with data for the remaining regions in Table 1.

TABLE 1. 1993/94 seasonal average *H. armigera* insecticide resistance levels at six monitoring locations in India (expressed as % survival at each discriminating dose).

Discriminating dose treatment	Northern region	Central region	S. Central Region	E. coastal region	Southern region	Extreme S. region
Cypermethrin 0.1 µg	77.1	78.2	83.2	95.6	85.2	76.3
Cyper 0.1+Pbo 50 µg	31.8	66.4	55.4	92.7	52.4	49.4
Suppression of resistance by Pbo	59.5	14.0	33.9	2.7	39.1	31.5
Cypermethrin 1.0 µg	23.9	23.9	44.5	70.8	52.0	-
Fenvalerate 0.2 µg	68.0	72.1	75.2	91.6	79.4	53.7
Endosulfan 10.0 µg	35.5	45.5	29.5	74.1	35.8	40.9
Quinalphos 0.75 µg	15.0	24.0	25.7	77.0	25.5	7.2

Pyrethroid resistance

Resistance both to cypermethrin and fenvalerate was high in all monitoring regions, with the situation being most severe (>80% survival at cypermethrin 0.1 µg) in Maharashtra, Andhra Pradesh and Tamil Nadu comprising the Central, South Central, Eastern coastal and Southern regions respectively (Figs 2 & 3).

In the Northern region the monitoring period was quite short (2.5 months), but there was a clear trend of increasing resistance with progression of the cotton growing season. SPs are still the pesticides of choice of farmers in this region for bollworm control, but in the latter half of the season these were proving to be ineffective and many farmers resorted to SP / OP mixtures. This region recorded the highest Pbo suppression of cypermethrin resistance at a seasonal average of 60% suppression.

In the Central region, SP resistance appeared to be bi-modal. Overall, both cypermethrin and fenvalerate resistance were high (averaging 78 and 72% survival respectively), with the lowest resistance levels (47 and 43% respectively) occurring mid season in October - November. For cypermethrin, the decrease in resistance correlated with a concomitant reduction in Pbo suppression which averaged 38% up to late October but only 4% for the remainder of the season. Similarly there was a marked change in profenofos suppressible SP resistance during the same period. Prior to the first week of October, profenofos suppression averaged only 6% and from November - mid March, 48%. The last collections in late March indicated significantly decreased suppression at 18%, most probably indicating a more important role for target site nerve insensitivity (*kdr*) resistance at this time.

In the Eastern coastal region the monitoring period was quite short this season for logistical reasons, but the data clearly shows that the Guntur cotton belt is still facing the most severe problem with >90% average seasonal survival at the SP discriminating doses. Even treatment at the 10x higher cypermethrin 1.0 μg dose gave an average of >70% survival. Pbo suppression was not significant at an average of only 2.7%.

The bimodal SP resistance trend observed in the Central region was also apparent in the Southern region at Coimbatore. However, here Pbo suppression remained fairly stable throughout the season, averaging 39% (range: 21-57%).

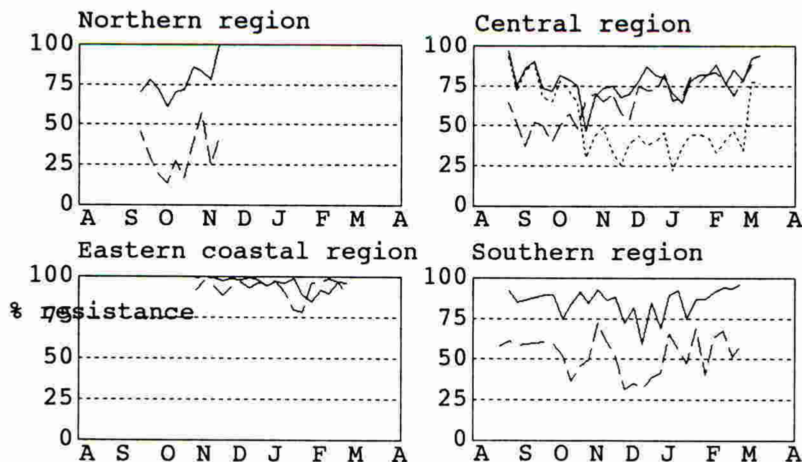

FIGURE 2. Average weekly resistance to cypermethrin (solid line) and effect of Pbo (broken line) and profenofos (dotted line), on suppression of cypermethrin resistance in *H. armigera* at four monitoring locations during the 1993/94 cropping season (based on % of 30-40 mg larvae surviving the cypermethrin 0.1 μg, cypermethrin 0.1 μg + Pbo 50 μg and cypermethrin 0.1 μg + profenofos 0.1 μg discriminating doses).

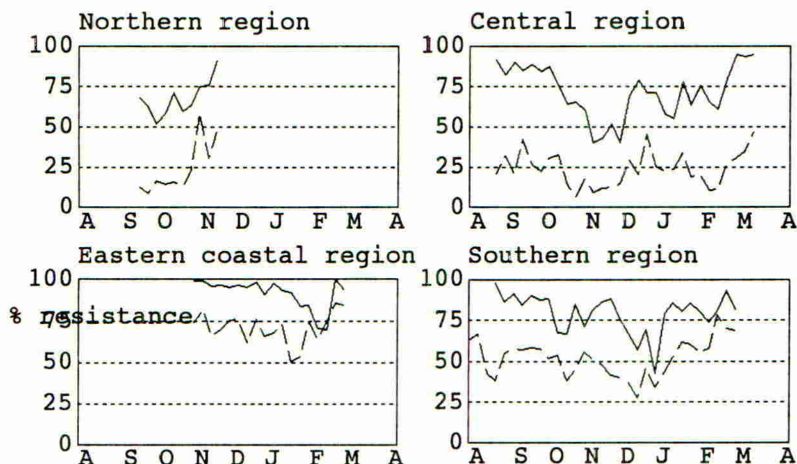

FIGURE 3. Average weekly resistance in *H. armigera* at four monitoring locations during the 1993/94 cropping season to the fenvalerate (solid line) and 10x LD_{99} cypermethrin (broken line) doses (based on % of 30-40 mg larvae surviving the fenvalerate 0.2 μg and cypermethrin 1.0 μg discriminating doses).

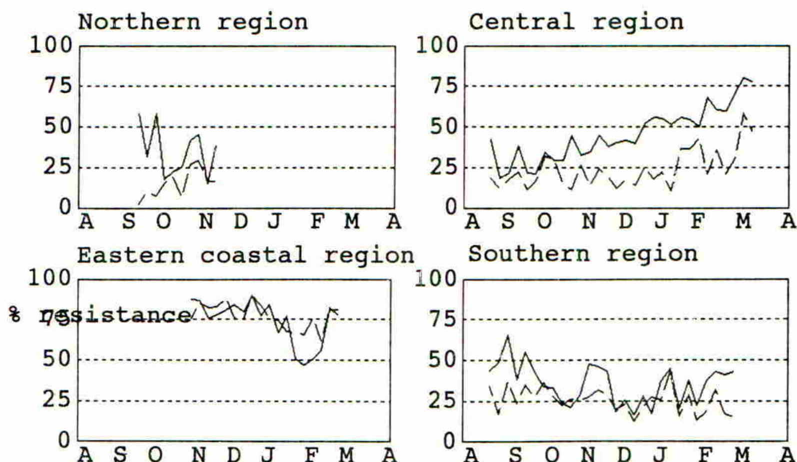

FIGURE 4. Average weekly resistance to cyclodienes (solid line) and organophosphates (broken line) in *H. armigera* at four monitoring locations during the 1993/94 cropping season (based on % of 30-40 mg larvae surviving the endosulfan 10 μg and quinalphos 0.75 μg discriminating doses).

<u>Endosulfan resistance</u>

Resistance to endosulfan averaged 36% in the Northern region and a seasonal decline was indicated. This probably arose because it was only widely used during the early season in Haryana, farmers preferring to use SP / OP mixtures later in the season as these were considered to be more

effective against *H. armigera*. In the Central region there was a clear trend of increasing resistance over the season (from 19% in September to 80% in March). In the Eastern coastal region, resistance was very high with a mean of over 74%. Resistance decreased from November to January and then increased again in February-March. In the Southern region, endosulfan resistance was highest early season (mean 49%), decreasing by early November to a fairly stable level (mean 32%) for the remainder of the season.

Organophosphate resistance

With the exception of the Eastern coastal region, moderate to low levels of resistance to quinalphos were recorded. Interestingly, in the Northern region, *H. armigera* was almost fully susceptible to quinalphos in September (<3% survival at quinalphos 0.75 μg), but had reached 30% resistance by mid November. Similarly in the extreme Southern region of Madurai, quinalphos resistance averaged only 7% and in several weeks 0-5% survival was observed. In the Central region, OP resistance remained fairly stable from September to January (mean 19%), but then increased markedly from then on to March (mean 37%). At Coimbatore in the Southern region, resistance remained fairly stable throughout the season (mean 26%, range: 13-36%).

DISCUSSION

The results clearly indicate that insecticide resistance in *H. armigera* is ubiquitous in India and can no longer be considered as a problem confined to the Central and Eastern coastal regions. Gene flow resulting from migration of moths from high input farming regions to areas where insecticide selection pressure is much less, is most likely to be responsible for the apparent homogeneity of resistance to SPs, cyclodienes and OPs over large areas of India (Armes *et al.*, 1992), and as reported by Daly and Gregg (1985) in Australia. Temporal differences in changes in resistance frequencies between regions are a reflection of regional variations in insecticide use and their effects on selection of resistant larval phenotypes over one or more locally breeding generations. To illustrate these points, Pbo and profenofos suppression of pyrethroid resistance provide good examples.

In the Eastern coastal region, where high insecticide inputs on cotton in particular are common (15-30 applications), Pbo synergism was not significant. The overuse of insecticides and SPs in particular appears to have resulted in strong genetic selection for nerve insensitivity (*kdr*) (West & McCaffery, 1993) as a significant mechanism, a situation which arose in Australia prior to implementation of a resistance management strategy (Gunning *et al.*, 1991). The Northern region (also an intensive cotton growing region), contrasts with the Eastern coastal situation in that insecticide inputs are in most seasons significantly lower in the north (6-12 applications), and the consequence of this appears to be high SP resistance but with a more significant role for mfo metabolism and probably (although untested) a lesser role for *kdr*.

In the Southern region, where insecticide use is highly variable (depending largely upon farmers' economic status), resistance is intermediate with moderate Pbo suppression and the likelihood of low frequencies of *kdr* resistance (West & McCaffery, 1993). It is quite feasible that 'injection' of highly resistant phenotypes by migration from the Eastern coastal region to the Central and Southern regions occurs during October-December at a time when the emergence of *H. armigera* moth populations is highest from cotton crops (Pedgley *et al.*, 1987; Armes *et al.*, 1992).

Profenofos synergism was tested only in the Central region. A 'switch' in metabolic mechanisms from significant mfo metabolism to esterase metabolism within one week implies a very rapid change in resistance genotypes. The bioassays were supported by quantitative estimates of cytochrome P450, carboxyl esterase and glutathione transferase activity indicating similar changes (Kranthi, *unpbl.*). It is difficult to account for such rapid change in terms of changes in local selection as there was no obvious shift in farmers' control tactics toward different insecticide chemistries at this time. A plausible explanation would be an influx of moths from populations subject to different

selection pressures contributing significantly to the gene pool (e.g. Daly, 1993), but we currently have no field evidence to confirm this. Levels of OP resistance were highly variable but significant across regions, probably largely as a result of different ways farmers were using these chemicals in cotton pest management. For example, in the North, endosulfan, SPs and monocrotophos (and their combinations) were the favoured chemicals for early season pest control and quinalphos resistance was low at this time. As control with these proved ineffective, there was a switch to quinalphos and chlorpyrifos in combination with SPs in the latter half of the season and this correlates with increasing quinalphos resistance at this time. Similarly, in the Central region, quinalphos resistance remained fairly stable up to January when OPs and endosulfan were the predominant choice for *H. armigera* control on chickpea by farmers.

For endosulfan, mean resistance was high across all regions and we frequently observed decreasing resistance over the early part of the cropping season. This probably arose because endosulfan is a popular early season insecticide (frequently popularised by agriculture departments as a good early season insecticide as it is considered relatively benign to natural enemies), being largely substituted later on by SPs and OPs.

Clearly the mechanisms involved and dynamics of insecticide resistance in *H. armigera* in India are complex, and for SPs at least, differs markedly from the routinely monitored situation in E. Australia where the implementation of a management strategy has moderated resistance toward a single locus mfo gene (Daly, 1993). Variable levels of selection pressure through unrestricted and inappropriate use of insecticides has resulted in selection of multiple resistance mechanisms and intractable resistance-related control problems over large areas of India. The use of agrochemicals in cotton pest management needs to be addressed urgently if *H. armigera* resistance is to be managed in the subcontinent.

ACKNOWLEDGEMENT

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INTERACTION OF CHLORDIMEFORM WITH MALATHION IN RESISTANT AND SUSCEPTIBLE *TRIBOLIUM CASTANEUM*

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ABSTRACT

The interaction of the acaricide, chlordimeform, and the organophosphorus insecticide, malathion, was investigated using two malathion resistant and two susceptible strains of *Tribolium castaneum* (Herbst), a flour beetle of economic importance. One of the strains was specifically resistant to malathion. On this latter strain, chlordimeform was highly synergistic to malathion, increasing the activity of malathion by 45 times at a ratio of malathion to synergist of 1:80. However, at higher ratios the synergistic effect disappeared. This strain was not synergised by piperonyl butoxide. With the two susceptible and the malathion non-specific resistant strains, there was synergism with PBO but antagonism with chlordimeform. The mechanism of these interactions were studied through *in vivo* enzyme analyses. A significant increase in carboxylesterase activity was observed with the synergism by chlordimeform. The cytochrome P_{450} levels were unaffected.

INTRODUCTION

Chlordimeform, N-(4-chloro-O-tolyl-N,N-dimethyl formamidine), (CDF) is an acaricide, which is particularly effective against eggs and neonate larvae. Very little is known of the mode of action of CDF against insects. Singh *et al.* (1981) reported that CDF and desmethyl-chlordimeform induced release of hyperlipaemic hormone which controls neuro-transmission at the synaptic junctions. Lund *et al.* (1978) reported that CDF could excite non-cholinergic synapses in the central nervous system to induce the light organs in the firefly, *Photinus pyralis* to glow. Thus CDF appears to bring about different responses in different insects.

Plapp (1979), El-Sayed & Knowles (1984) and Fisher (1992) reported that although CDF is not toxic to most insects in combination with certain insecticides belonging to the organophosphorus, pyrethroid and carbamate classes, CDF increased toxicity manyfold. We report here results of experiments on the interaction of malathion and CDF in malathion resistant and susceptible strains of adult *Tribolium castaneum*.

MATERIALS AND METHODS

Four strains of *Tribolium castaneum* were used in the bioassays. *T. castaneum* strain CTC-12 is resistant to malathion and other organophosphorus insecticides (Champ &

Campbell-Brown, 1970) while strain FSS II is susceptible to insecticides (Lloyd & Ruczkowski, 1980). Strain Kano-C is resistant only to malathion (Dyte & Rowlands, 1967) while strain CR-1 is slightly more resistant to malathion than FSS II.

Insects were reared in sterilised jars containing whole wheat flour with yeast. The jars were kept in an incubator at 70% relative humidity and 30°C with a diurnal of 12:12 dark:light cycle. Under these conditions adults emerged in about 28 days. All bioassays were carried out with adult insects of 8 to 10 days old. CDF (93% pure) and malathion (98% pure) were of technical grade.

Treatment of insects and determination of toxicity

Glass petri-dishes of 20 cm^2 internal bottom surface area were treated with predetermined acetone solutions of malathion or chlordimeform and mixtures of malathion and chlordimeform. Insects (10) selected at random were placed in each covered petri-dish and left for 24 hours, at the end of which time insects that did not move when prodded gently with a soft brush were scored as dead. Probit analysis was done using the method of Busvine (1971). Cotoxicity coefficient values (LC_{50} for toxicant alone/LC_{50} of toxicant in the mixture x 100) for mixtures of malathion with CDF were calculated according to the method described by Sun & Johnson (1960).

Enzyme assays

Total and carboxylesterase (CarE) activities were measured according to the method of Devonshire (1977). Adult beetles were first exposed to those concentrations (of a residual film in petri-dishes) of each compound alone and of its mixture with a synergist that gave 5% mortality in 3 hours. This was to eliminate the most susceptible individuals and to encourage induction of enzymes. These insects were then exposed to concentrations that would kill 50% in 24 hours. In the case of mixtures only the highest ratio that showed the highest synergistic effect was used. After 24 hours of exposure to the compound or its

TABLE 1. Lethal concentrations (LC_{50}) for four strains of adult beetles of *Tribolium castaneum*.

Strain	LC_{50} ($\mu g/cm^2$)	95% confidence limit lower	upper	slope	intercept
Malathion:					
CTC-12	0.0164	0.013	0.020	1.537	3.13
Kano-C	2.2235	1.828	2.703	2.857	4.01
FSS-11	0.0019	0.001	0.002	3.728	3.96
CR-1	0.0073	0.007	0.008	9.585	-3.32
Chlordimeform:					
CTC-12	14.92	14.20	15.67	8.52	5.10
Kano-C	9.27	7.03	12.22	3.75	1.40
FSS-11	4.30	3.69	5.01	3.74	2.63
CR-1	4.05	3.08	5.34	3.65	2.77

mixture the insects that survived were weighed and homogenised in 0.01M phosphate buffer (pH 7.0) for total esterase measurements and in phosphate buffer with eserine sulphate (0.1M) for CarE activity. The homogenate was filtered through glass wool and esterase activity measured using a substrate of 1-napthyl acetate (nM hydrolysed per mg insect soluble protein per hour), based on the linear relationship of activity with time.

Cytochrome P_{450} activity was measured in surviving insects according to Omura & Sato (1964). Cytochrome P_{450} was determined from the difference in spectra (OD at 490-500 nm) between the sodium dithionite reduced enzyme and the CO complex, using a molar extinction coefficient of 91 $nMcm^2$. Protein measurements were by the method of Lowry *et al.* (1951).

TABLE 2. Cotoxicity coefficient values for mixtures of malathion with chlordimeform in four strains of *T. castaneum*.

	ratio by weight mal:CDF	LC_{50} of mixture ($\mu g/cm^2$)	concentration ($\mu g/cm^2$) in mixture of: mal	CDF	cotoxicity coefficient
CTC-12:	5:1	0.072	0.060	0.012	26.20
	10:1	0.079	0.072	0.007	22.84
	1:1	0.047	0.024	0.024	69.20
	1:5	0.103	0.017	0.085	95.91
	1:10	0.032	0.029	0.294	55.93
	1:20	0.610	0.029	0.581	56.36
Kano-C:	5:1	4.035	3.363	0.673	66.12
	1:1	3.055	1.528	1.528	145.53
	1:10	2.816	0.260	2.601	854.86
	1:40	3.373	0.082	3.290	2701.70
	1:80	3.724	0.050	3.592	4468.84
	1:120	232.320	1.920	230.420	116.40
	1:140	315.150	2.224	311.290	100.00
	1:160	357.980	2.224	355.760	100.00
FSS-II:	5:1	0.006	0.005	0.001	36.54
	1:1	0.023	0.012	0.012	16.59
	1:5	0.026	0.004	0.021	45.24
	1:10	0.173	0.016	0.157	12.10
	1:20	0.624	0.030	0.594	6.40
CR-1:	5:1	0.087	0.072	0.014	10.14
	10:1	0.018	0.016	0.002	44.86
	1:1	0.233	0.012	0.012	62.66
	1:5	0.228	0.038	0.109	19.21
	1:10	0.177	0.016	0.160	45.63
	1:20	0.864	0.041	0.823	17.76

mal = malathion; CDF = chlordimeform

RESULTS AND DISCUSSION

Malathion was toxic to all four strains of *T. castaneum* (Table 1). CDF was also toxic to all four strains but toxicity was much lower compared with malathion (Table 1). Of the two resistant strains Kano-C strain was more resistant to malathion than strain CTC-12. However, with CDF the opposite was true, in that CTC-12 was more resistant to CDF than Kano-C strain.

The two susceptible strains (FSS-II and CR-1) and one of the resistant strains (CTC-12) showed antagonism between malathion and CDF, while the Kano-C strain showed synergism (Table 2). An interesting phenomenon observed was that where synergism occurred with CDF (Kano-C) synergism did not level off as would be expected with a conventional synergist such as piperonyl butoxide (PBO). On the contrary, synergism began to decline after reaching a peak to a level indicating neither synergism nor antagonism (Table 2). Thus, CDF does not appear to behave in a similar way to PBO.

TABLE 3. Cotoxicity coefficient values for mixtures of malathion with piperonyl butoxide (PBO) in *Tribolium castaneum.*

Strain treatment	ratio by weight	LC_{50} of mixture ($\mu g/cm^2$)	concentration of compounds in mixture: mal	PBO	cotoxicity coefficient	SR
CTC-12						
	5:1	0.016	0.013	0.003	126	1.0
	1:1	0.015	0.008	0.008	205	1.1
	1:2	0.022	0.007	0.015	234	0.7
	1:5	0.003	0.007	0.037	234	0.5
	1:10	0.080	0.007	0.073	234	0.2
Kano-C						
	5:1	2.662	2.223	0.446	100	0.84
	1:1	4.446	2.223	2.223	100	0.10
	1:5	13.344	2.224	11.120	100	0.17
	1:10	24.431	2.224	22.210	100	0.09
FSS-11						
	5:1	0.006	0.001	0.005	190	0.32
	1:1	0.003	0.002	0.002	127	0.63
	1:5	0.007	0.001	0.006	190	0.27
	1:10	0.013	0.001	0.012	190	0.15
CR-1						
	5:1	0.006	0.005	0.001	146	1.20
	1:1	0.012	0.006	0.006	121	0.60
	1:5	0.036	0.006	0.033	121	0.20
	1:10	0.066	0.006	0.060	121	0.10

PBO = piperonyl butoxide, mal = malathion
SR = synergistic ratio (unsynergised LC_{50}/synergised LC_{50})

There was no synergism or antagonism between piperonyl butoxide (PBO) and malathion in the Kano-C strain of *T. castaneum*. But with the two malathion susceptible strains (FSS-11 and CR-1) and the other malathion resistant strain (CTC-12) PBO synergised malathion (Table 3). The results with Kano-C strain agree with those of Dyte & Rowlands (1967) who reported that PBO is inactive as a synergist of certain organophosphorus insecticides in Kano-C strain. They suggested that the reason for synergism in one strain and antagonism in others may be due to different detoxification mechanisms.

Enzyme assays carried out with the strain of *T. castaneum* where CDF synergised malathion (Kano-C) (Table 4) showed that CarE activity was significantly increased compared to the control. However, cytochrome P_{450}, the mixed function oxidase (MFO), associated with detoxification of xenobiotics showed no significant difference between treatment and control (Table 5). Scott *et al.* (1985) reported that monoamine oxidase (MAO) is sensitive to CDF. However, with this particular strain of the flour beetle it appears that P_{450} does not play any role in this interaction.

TABLE 4. Esterase activity *in vivo* of insects treated with CDF as a synergist combined with malathion in Kano-C strain.

Treatment	Esterase activity		(μM/h/mg protein)	
	CarE	sd	esterases	sd
Control	2.17 b	0.06	3.10 b	0.06
Malathion (Mal)*	1.56 a	0.02	2.10 a	0.05
Chlordimeform (CDF)*	2.19 b	0.05	3.04 b	0.08
Mal:CDF (1:80)*	2.56 c	0.06	3.02 b	0.05

* treatments at LC_{50} level; sd = standard deviation; CarE = carboxylesterase; Mean of 16 replicates. In columns means followed by a common letter are not significantly different (P < 0.05) (Duncan's Multiple Range Test).

TABLE 5. Effect on cytochrome P_{450} content of malathion with chlordimeform and piperonyl butoxide (PBO) in *T. castaneum* Kano-C strain.

Treatment	Cytochrome P_{450} content and sd (nM/mg protein)	
Control	0.16 a	0.01
malathion*	0.16 a	0.02
Chlordimeform (CDF)*	0.17 a	0.03
malathion:CDF (1:80)*	0.17 a	0.01
malathion:PBO (1:10)*	0.16 a	0.02

* at LC_{50} level; sd = standard division; Mean of 10 replicates; In columns means followed by a common letter are not significantly different (P < 0.05) (Duncan's Multiple Range Test); PBO = piperonyl butoxide.

As CDF synergised malathion in one malathion resistant strain and antagonised another resistant and two susceptible strains its application both as an acaricide and as a synergist should be carefully monitored, especially in mixed populations of insect pests in order to prevent the building up of resistance to insecticides (Fisher, 1992).

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THE DEVELOPMENT OF A SAME-DAY TEST FOR DETECTING RESISTANCE TO PHOSPHINE AND ITS APPLICATION TO FUMIGATION STRATEGIES

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ABSTRACT

A new test to identify resistance has been developed for three species of stored product beetles and is presently being developed for a fourth, based on knockdown responses of adults on paper cones during exposure to phosphine. Adults of the susceptible strains of *Cryptolestes ferrugineus* (Stephens), *Tribolium castaneum* (Herbst) and *Oryzaephilus surinamensis* (L.) were all knocked down within 3 h and those of *Sitophilus oryzae* (L.) within 4.5 h in 0.31 - 0.42 mg/l phosphine. Homozygous resistant adults of *C. ferrugineus, T. castaneum* and *S. oryzae* remained active for more than 20 h with no overlap between the responses of susceptible and homozygous resistant populations. Individuals of *O. surinamensis* also remained standing after 20 h although these were not from populations consisting wholly of homozygous resistant insects. There was no overlap of the regression lines of the reference insects of *O. surinamensis* and insects from a population selected for resistance with 0.39 mg/l of phosphine, nearly eight times the F.A.O. discriminating dose for this species.

An F_1 generation was obtained for *C. ferrugineus, T. castaneum* and *S. oryzae* by pooling the progeny of single pair crosses of homozygous resistant and susceptible individuals. The knockdown responses gave regression lines intermediate between susceptible and resistant populations for each species lying close to those of the susceptible population. There was considerable overlap between susceptible and heterozygous populations but it was still possible to set discriminating exposure times, based on total knockdown of susceptibles, as follows: *C. ferrugineus,* 2 h 40 min at 0.36 $\pm$ 0.05 mg/l; *S. oryzae,* 4 h 32 min at 0.39 $\pm$ 0.03 mg/l; *T. castaneum,* 2 h 42 min at 0.38 $\pm$ 0.04 mg/l. Heterozygotes of *O. surinamensis* have yet to be tested but the reference data indicates a discriminating time of 2 h at 0.42 $\pm$ 0.02 mg/l.

For two of the species, *T. castaneum* and *C. ferrugineus,* strains recently collected from provender mills in the U.K.were tested using the standard F.A.O. mortality test and these results were subsequently compared with those obtained using the new test method. There was excellent agreement between the two tests.

INTRODUCTION

Since the F.A.O. survey of pesticide resistance in stored product pests in the 1970s (Champ & Dyte, 1976), the potential for problems arising from resistance to phosphine has been well known. Initially the levels of phosphine resistance detected were quite low, both in terms of incidence among different populations and in terms of the extent to which tolerance levels were increased among individual beetles. During the 1980s, however, very much higher tolerances were encountered among resistant populations, firstly in Bangladesh and India (Tyler *et al.*, 1983; Taylor, 1986), and later in parts of Africa, South America and Indonesia (Taylor, 1989). Subsequent tests revealed that such populations also displayed resistance in their naturally tolerant immature stages and that there were serious implications for control (Price & Mills, 1988; Mills *et al.*, 1990). Furthermore, it has been suggested that high levels of resistance can result from selection with phosphine at any stage of the life cycle (Rajendran,1992).

Current fumigation practices require high standards of sealing and dosing. If these are not achieved there is an inherent risk of selection for resistance. In addition, there is potential for the future use of phosphine to increase substantially due to the possible loss of methyl bromide because of its suspected link with the destruction of stratospheric ozone (Chakrabarti & Bell, 1993). A rapid resistance test would be a vital aid in minimising the risk of selection for resistance because it would allow adequate time for an appropriate fumigation strategy to be formulated.

METHODS

Three genotypes, homozygous susceptible, homozygous resistant and heterozygous resistant insects of all the species were tested except for *O. surinamensis* where a homozygous resistant population and a heterozygous population were not yet available for testing. For this species the homozygous susceptible insects were tested alongside insects from a genetically mixed population and from the same population which had been selected once with 0.39 mg/l phosphine. The insects in all cases were from mixed-age populations. The susceptible insects were taken from laboratory stock cultures previously established as true-breeding susceptible populations using the F.A.O. test. The homozygous resistant insects were from populations previously selected for phosphine resistance as virgin adults using high concentrations and the F.A.O. methodology. Heterozygotes were produced from single-pair crosses between virgin adults of the reference strain and the homozygous strain. Pairs were set up over several consecutive weeks and the progeny from all the pairs then combined. Every 3-4 weeks, depending on the life-cycle of the species, the heterozygotes were transferred to fresh food to prevent the emergence of an F_2 generation containing other genotypes.

The insects were tested on paper cones (6.5 cm diameter, 3 cm high) in glass crystallising dishes (7 cm diameter, 4 cm deep) sealed at the base with molten paraffin wax to prevent the insects from getting under the edge of the paper. For glass-climbing insects the walls of the dishes were coated with a thin coating of an aqueous suspension of polytetrafluoroethylene to prevent them from escaping. Tests were conducted in 6-litre glass desiccators, fitted with a

flat glass top to enhance observation of the insects. The top contained a central dosing port fitted with a rubber septum.

To establish the discriminating times 3 replicates of 20 insects were observed for each strain. The dishes were placed on a wire-mesh shelf inside the desiccator, approximately 6 cm from the top, and left to acclimate in the test conditions of 25° C, 60% r.h. for at least 30 min before sealing with the glass top. The desiccators were then dosed with phosphine, generated using the F.A.O. test method (Anon., 1975), using a gas-tight syringe through the central dosing port and then stirred with a magnetic stirrer for approximately 5 min. Timing was started from the introduction of fumigant. The insects were observed at intervals of 2-5 min until all the insects were knocked down. Knocked-down individuals were defined as those unable to retain their grip on the sloping side of the cone and which fell to the bottom and were unable to stand.

Subsequently, a number of field strains of *T. castaneum* and *C. ferrugineus*, obtained from provender mills in the U.K., were tested for resistance using the F.A.O. mortality-based resistance test. These strains were then tested using the new method. Initially each test was observed every 2-5 min as before (exploratory test) and then three replicates of 50 insects were left for a fixed period, based on the estimated discriminating knockdown time and then assessed for knockdown (discriminating test). In both tests each field strain was tested alongside the susceptible strain. At the end of each test the concentration of phosphine in the desiccators was analysed by gas chromatography, using a flame photometric detector, calibrated with a cylinder formulation of phosphine in nitrogen of known concentration. After the tests were completed the knockdown data were subjected to probit analysis (Finney, 1971). In order to set a discriminating time for each species the data for the susceptible strains, obtained within the stated concentration range, was pooled and the value for 99.9% knockdown ($KD_{99.9}$) was used as an estimate.

RESULTS

The probit regression lines for *C. ferrugineus* and *T. castaneum* showed a clear distinction between the homozygous susceptible and the homozygous resistant insects with at least 12 h between the last susceptible insect and the first resistant insect being knocked down (Figs. 1 and 2). The distinction was equally clear for *S. oryzae* with the last susceptible insect knocked down after 4 h 8 mins and the first homozygous resistant individual knocked down after 13 h, estimated from the probit response line (Fig. 3). The present data for the susceptible strain of *O. surinamensis* show partial overlap with the responses of the unselected population, which contained all genotypes, and no overlap between the population selected with 0.39 mg/l phosphine using the F.A.O. test methodology (Fig. 4). The regression lines for this species indicate that there will be no overlap between the homozygous resistant line and the susceptible line. The regression lines for the heterozygotes, where tested, all lay between those of the susceptible strain and those of the resistant strain albeit very near to the susceptible lines (Figs. 1 and 2). There was considerable overlap in the response times of the susceptible and heterozygote populations, although for *T. castaneum* and *C. ferrugineus* there was always at least 1 h between the last susceptible insect and the last heterozygote insect being knocked down. The results for *S. oryzae* (Fig. 3) showed less consistency among replicate tests and

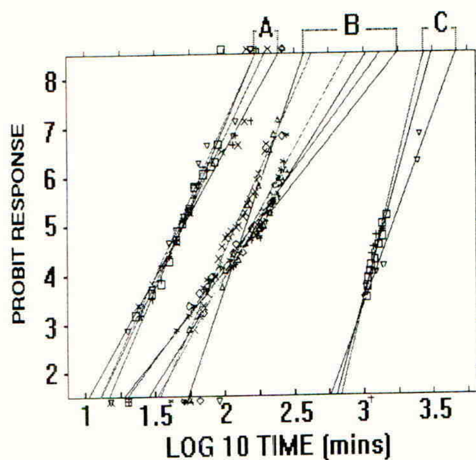

FIGURE 1. Knockdown responses of three genotypesof *T. castaneum* at concentrations of 0.4 ± 0.03 mg/l phosphine.

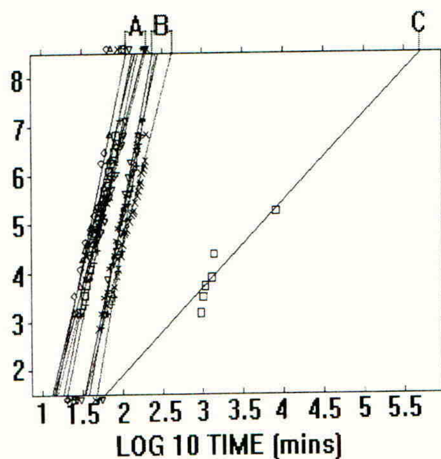

FIGURE 2. Knockdown responses of three genotypes of *C. ferrugineus* at concentrations of 0.36 ± 0.05 mg/l phosphine.

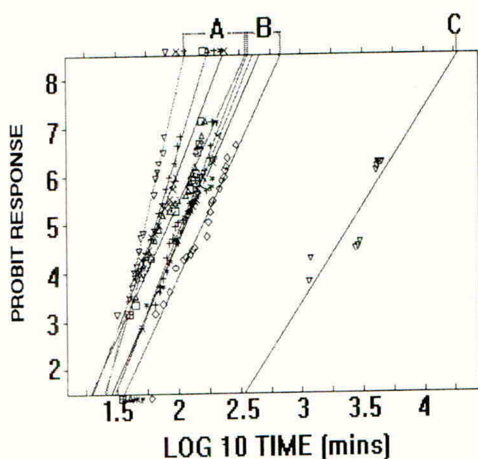

FIGURE 3. Knockdown responses of three genotypes of *S. oryzae* at concentrations of 0.39 ± 0.03 mg/l phosphine: C = 0.46 mg/l.

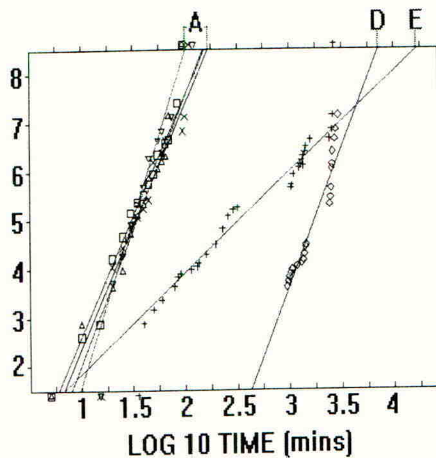

FIGURE 4. Knockdown responses at 0.42 ± 0.01 mg/l phosphine of the reference strain of *O. surinamensis*,an unselected population of a resistantstrain and the same resistant strain selected with 0.39 mg/l phosphine.

KEY:
A = homozygous susceptible
B = heterozygotes
C = homozygous resistant
D = resistant strain after selection with 0.39 mg/l phosphine
E = unselected resistant strain

there was a greater degree of overlap between heterozygote and susceptible strain results than for the other two species.

The field strains of *T. castaneum* identified as resistant by the F.A.O. test were also shown to be resistant by the knockdown test and all but one strain had responses lower than that of the mean susceptible response. This strain collected from Northfields, gave inconsistent results in both tests. Initially, one survivor was recorded in the F.A.O. test and the strain was classified as resistant. However, a re-test showed no resistance. In the knockdown test no insects remained standing in the exploratory test but one insect remained standing at the end of the discriminating test. The regression parameters for the field strains from the exploratory tests are given in Table 1.

TABLE 1. Regression parameters for field strains of *T. castaneum* and *C. ferrugineus* tested with the new test

Susceptible	KD_{95} (fiducial limits)	SLOPE (S.E.)	Resistant	KD_{95}	SLOPE
T. castaneum:					
Susceptible	91.24 (86.9, 96.5)	5.8 (0.2)	Calne	176.5 (157.4, 202.9)	3.9 (0.2)
Canterbury	59.5 (55.8, 64.3)	7.0 (0.4)	Chard	448.7 (349.9, 642.5)	2.8 (0.3)
Earls	108.0 (96.1, 126.8)	6.1 (0.6)	Melksham	324.6 (278.2, 392.7)	3.0 (0.2)
Thorpe	114.1 (102.2, 133.9)	6.7 (0.7)	Wrexham	400.6 (327.5, 519.6)	2.9 (0.2)
Northfields	71.3 (67.8, 75.7)	9.1 (0.6)	Wells	377.4 (295.3, 528.6)	2.9 (0.2)
C. ferrugineus:					
Susceptible	93.5 (88.8, 99.2)	6.2 (0.3)	Shifnal	103.6 (92.0, 122.0)	5.4 (0.5)
Milton	70.5 (66.6, 75.6)	7.6 (0.5)	Kinross	98.4 (92.4, 106.1)	5.8 (0.3)
Biggleswade	72.3 (67.4, 78.9)	6.7 (0.4)			

DISCUSSION

Previous attempts to identify resistance to phosphine, based on the ability of insects to remain active under gas, have been hampered by the difficulty of determining whether or not insects have become immobilised and the complication of narcosis at high gas concentrations. Reichmuth (1992), for example, tested adults of *Rhizopertha dominica* (F.) at concentration levels well above the 0.4 mg/l tested here and assessed resistance on the basis of insects not becoming 'motionless' or 'narcotised' within about half an hour. Narcotic concentration thresholds have not yet been identified for this species but it is known that narcotic thresholds for susceptible and resistant strains of *T. castaneum* differ widely. The threshold for the susceptible strain lies near 0.5 mg/l (Winks, 1985) with a 10-fold increase for a resistant strain (Winks & Waterford, 1986).

The uncertainty associated with the narcotic responses of insects and the concentration levels at which they occur indicate that a resistance test based on narcotic response would be hard to interpret . For each species, consideration would have to be given to variation of narcotic response among strains and among genotypes within a strain. For these reasons the current study is based on a knockdown response at concentrations below that which initiate

narcosis in susceptible *T. castaneum*. Knockdown is more clearly distinguished from the normal movements of an insect and the use of paper cones renders the assessment of knockdown much easier. The number of test insects was initially only 20 per replicate for the experimental observations which provided the regressions of knockdown against time. This was in order to facilitate accurate observation to obtain the provisional discriminating knockdown times though no limit need be place on the number of individuals in a discriminating dose test. A larger sample size would enable the detection of low incidences of resistant individuals.

A good agreement was obtained between the results of the current test method and those of the F.A.O. discriminating dose test for *T. castaneum* and *C. ferrugineus*. In strains where the incidence of resistant insects in the population was in the order of 1%, both tests were equally efficient at detecting resistance. Thus these preliminary results indicate that the new test method is likely to be as efficient as the F.A.O. test in identifying resistance . It has the considerable advantage of being able to give a result within a working day so that the findings can be used to formulate a fumigation strategy. It has also made it possible to differentiate heterozygous insects from susceptible ones, provided that a sufficient number of heterozygotes are present in the population sample at the time of testing. This gives the added advantage of being able to detect the presence of resistance in a population at a relatively early stage in the selection process before the homozygous genotype becomes established in response to inadequate phosphine fumigations. In order to make full use of the advantages presented by this test more information is required on the mortality responses of all life stages of heterozygous resistant insects.

At present only three species have been fully tested using the new method. Further tests are required on these with a larger number of strains, both resistant and susceptible when diagnosed by the F.A.O. method, to establish a more reliable discriminating time between resistance and susceptibility. Attempts are currently being made to improve the differentiation between the responses of susceptible and heterozygous *S. oryzae* populations by experimenting with different phosphine concentrations. The data for *O. surinamensis* suggests that further tests will show a distinction between the three genotypes. A breeding and selection programme is underway to create a homozygous resistant population of *O. surinamensis* with a view subsequently to producing a heterozygote population for testing. It is clearly desirable that the test method is investigated against other species for which an F.A.O.-based discriminating dose test is available.

In spite of growing resistance problems, phosphine is still effective in achieving control provided that adequate gas concentrations can be maintained so that the exposure period can be extended. For this to be possible, the storage structure needs to be sealed to pressure test standards (Banks & Annis, 1980) or there needs to be some means of maintaining a constant gas concentration throughout a storage structure for the necessary exposure period (Winks, 1990). The latter is possible with the continuous flow systems developed in Australia and the U.K. which utilise a cylinder-based supply of phosphine in carbon dioxide.

With continuous flow systems it is possible to extend an exposure period to any chosen duration, although this may increase treatment costs. It would be of considerable value if the resistance status of the pest population was known at the start of a treatment so that the need for any special measures could be ascertained. At present the discriminating dose test for

common beetle species relies on a 14-day mortality assessment following a 20-h exposure to phosphine. A 15-day delay between discovery of an infestation and arranging a treatment is often unacceptable and information on resistance is unlikely to be obtained in time to influence the control strategy.

The possibility that resistance may be identified by the different activity levels of individuals under gas, a likely side effect of reduced uptake or active exclusion (Price, 1984), has already been partly explored (Reichmuth, 1992). In the current study a rapid diagnostic test has been developed for some common beetle pests by which the presence of resistance in populations can be identified within a few hours.

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THE POTENTIAL FOR INDUCED EXTRINSIC HOST PLANT RESISTANCE IN IRM STRATEGIES TARGETING THE DIAMONDBACK MOTH

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ABSTRACT

Insecticide resistance has posed an immense obstacle to effective control of the diamondback moth, *Plutella xylostella* for over forty years, particularly in South East Asia. Within the last decade, products based on *Bacillus thuringiensis* Berliner (*Bt*) have been used increasingly and have shown greater compatibility with natural enemies of *P.xylostella* compared with conventional insecticides, although resistance to *Bt* is now increasing. This study provides recent evidence of resistance by Malaysian field strains of the diamondback moth to *Bt* products and abamectin. It then examines the use of intermediate levels of host plant resistance to enhance effective parasitism by endolarval parasitoids. Such plant resistance may be intrinsic or induced by application of low doses of secondary plant products.

INTRODUCTION

Plutella xylostella L., the diamondback moth, is the key crop protection problem on Cruciferae in South East Asia. Since the 1950s, attempts to control *P.xylostella* have been characterised by its ability to develop resistance rapidly to successive groups of insecticides (Talekar, 1992). Over the past decade, farmers in the region have been substituting broad spectrum compounds with more selective products, particularly those based on *Bacillus thuringiensis* Berliner (sspp *kurstaki* and *aizawai*) [*Bt*] (Loke et al., 1992; Ooi, 1992). The observed population build-up of several parasitoid species, notably *Cotesia plutellae* Kurdj. (Hymenoptera: Braconidae) and *Diadegma semiclausum* Horst. (Hymenoptera: Ichneumonidae), in the Cameron Highlands of Malaysia, may have been related to such changes in pesticide usage (Furlong et al., 1994). However, there is now resistance to *Bt* products and other selective compounds such as acylureas in populations of *P.xylostella* in Malaysia and elsewhere (Furlong et al., 1994; Tabashnik, 1994). There is therefore an urgent need to devise alternative strategies for insecticide resistance management (IRM). In the present study, certain host plant/cultivar/age groups have been shown to enhance effective parasitism of *P.xylostella* in the laboratory. However, as market forces do not generally encourage farmers to select plant cultivars with intrinsic host plant resistance (van Emden & Way, 1972; Loke et al., 1992), further studies (laboratory and semi-field) are described testing the induction of extrinsic resistance (Price, 1986) by application of low doses of a neem seed kernel extract (NSKE) to *P.xylostella*-susceptible cultivars. Such methods have potential as a tool for the improvement of biological and integrated control, whilst avoiding problems caused by intensive insecticide use.

MATERIALS AND METHODS

Plants

Chinese cabbage [cv. Tip Top], Chinese mustards [cvs Pak choy and Sawi manis] were grown organically in a glasshouse. Common cabbage cultivars used in laboratory experiments were outdoor-grown in pots. Experiments were conducted between July 1993 and August 1994. Host

plant/cultivar/age/growing method combinations used in tritrophic laboratory experiments were selected to provide high, intermediate and poor intrinsic nutritional quality to *P.xylostella* (Verkerk & Wright, 1994).

Test insects

Insect cultures and experiments were maintained in constant environment rooms at 20±2°C and 65±3% r.h. under a 16:8 L:D cycle. An insecticide-susceptible laboratory strain (ROTH) of *P.xylostella* was obtained from Rothamsted Experimental Station, UK; three field strains were collected in the Cameron Highlands, Malaysia (TR from Central Zone [Tanah Rata]; KT from Northern Zone [Kuala Terla] and HABU from Southern Zone [Habu]) in April 1994; and a fourth field strain (SERD 2) was collected from the Malaysian lowlands, near Serdang, Selangor State, in February 1994. Laboratory cultures of *D.semiclausum* and *C.plutellae* were used (see Furlong et al., 1994).

Chemicals

The neem seed kernel methanolic extract [EC] (2.2% wt/*V* azadirachtin) was obtained from the Institut Phytopathologie, Giessen, Germany. Other compounds tested were abamectin (avermectin B1; Agrimec®:18 µg AI/ml), *Bacillus thuringiensis* Berliner ssp *kurstaki* (Dipel®: 16000 iu/mg); and *B.thuringiensis* ssp *aizawai* (Florbac®:8500 iu/mg). Test solutions of pesticides were freshly prepared for assay in distilled water with Triton X-100 (50 µg AI/ml) as an additional surfactant.

Residual ingestion (leaf-dip) bioassays

A laboratory leaf-dip bioassay was used, as described by Fauziah et al. (1992).

Effect of host plant cultivars on parasitoid cocoon formation in the laboratory

Single, mated female parasitoids were introduced to Petri dishes (5 and 9cm diameter for *C.plutellae* and *D.semiclausum* respectively) containing leaf discs supporting second instar *P.xylostella* larvae (ROTH strain) which had been allowed to establish feeding for 2h. Parasitoids were removed from Petri dishes after 1h, a duration found to allow oviposition attempts in most, if not all larvae. A parallel experiment excluding parasitoids was set up for comparison.

Effect of a low dose (*c.* LC10) of neem seed kernel extract on *D.semiclausum* cocoon formation in the laboratory

The method was similar to that described for *D.semiclausum* above, except that Chinese cabbage leaf discs (9cm diameter) were used and parasitoid oviposition occurred on untreated discs. Leaf discs were dipped as described above in an emulsion of neem seed kernel extract (0.8µg azadirachtin/ml); control discs were untreated. Treated and untreated leaf discs were subsequently provided *ad lib.* as food sources for the neem-treated and control insects respectively.

Semi-field trial

Plants of three *P.xylostella*-susceptible host plant cultivars (Chinese cabbage cv. Tip Top and Chinese mustards cvs Pak choy and Sawi manis) were grown in the greenhouse for 52 days. The plants were then placed within a nylon mesh cage in the laboratory in a Latin square arrangement for oviposition (48h; ROTH strain). The plants were transplanted in a randomised block design in a field plot: 4 plants per treatment; 6 treatments per block; and 5 blocks. The plants were maintained in the field plot from 13 to 21 July 1994. The neem-treated plants were sprayed with an emulsion of neem

seed kernel extract (0.8μg azadirachtin/ml; 0.1% *V/V* Codacide® oil) one day prior to *P.xylostella* egg hatch (16 July) and on a second occasion when the majority of larvae were in the second instar (20 July), using a coarse spray from an adjustable cone nozzle of a pneumatic sprayer giving an approximate rate of 250 l/ha. The plants were harvested when the larvae had been in a parasitisable state for about 3 days and were immediately transferred to individual labelled plastic bags. All plants were destructively sampled; *P.xylostella* larvae being transferred to labelled culture boxes. Larvae were maintained with untreated or recently sprayed leaves (for control and treatment respectively) *ad lib.* Bionomic data were gathered after 12 days.

RESULTS

Relative toxicity of Agrimec®, Dipel® and Florbac® to *P.xylostella* field strains

The four field strains tested exhibited varying patterns of relative toxicity to Agrimec and the two *Bt* products. Resistance ratios [RR] (LC50 of given strain / that of ROTH laboratory strain) with Agrimec were greater for the Cameron Highland strains (maximum RR = 195.2 for KT) compared with the lowland strain (SERD 2 = 17.2). RR values for the *Bt* products were generally lower than for Agrimec and resistance to Florbac was negligible in all strains (maximum RR = 2.3 for SERD 2). Dipel resistance was appreciable in both SERD 2 and HABU (RR = 25.1 and 19.0 respectively).

TABLE 1. Tritrophic (host plant/cv./age group + *P.xylostella*[1] + *C.plutellae* / *D.semiclausum*) bionomic characters measured in the laboratory at experimental end-points (day 20)[2].

Parasitoid species Host plant and cultivar.	Percent			
	Exposed to parasitoid			Parasitoid-free
	No. cocoons formed / larvae exposed	*P.xylostella* survivors	*P.xylostella* larval mortality	*P.xylostella* mortality
C.plutellae				
Tip Top	29 a	32 a	39 a	7 a
Wheelers Imperial	52 b	8 b	40 a	15 a
Red Drumhead	44 b	14 b	42 a	18 a
D.semiclausum				
Tip Top	32 b	12 a	56 a	20 a
Wheelers Imperial	52 c	7 a	41 a	58 b
Red Drumhead	14 a	7 a	79 b	55 b

Values within columns and within species followed by a common letter are not significantly ($P > 0.05$) different (ANOVA and pairwise LSD-tests following arcsine transformation of percentage data; *C.plutellae*: 10 *P.xylostella* larvae/replicate, 8 replicates/treatment; *D.semiclausum*: 20 *P.xylostella* larvae/replicate, 4 replicates/treatment).

[1] ROTH laboratory strain

[2] End-points for *C.plutellae* experiment = July; For *D.semiclausum* = November

Effect of host plant cultivars on parasitoid cocoon formation in the laboratory

The proportion of cocoons formed by *C.plutellae* was significantly greater on *P.xylostella*, maintained on mature cvs. Wheelers Imperial and Red Drumhead than on young cv. Tip Top and accordingly, a larger proportion of *P.xylostella* survived to adulthood on the last cultivar (Table 1).
In contrast, the number of cocoons formed by *D.semiclausum* was greatest on cv. Wheelers Imperial, intermediate on cv. Tip Top and least on cv. Red Drumhead. Seasonal effects on plants contributed to differences in *P.xylostella* survival in the absence of parasitoids (Table 1).

Effect of a low dose of neem seed kernel extract on parasitoid cocoon formation

The results showed that slight nutritional stress imparted by the low dose of neem extract could enhance effective parasitism (proportion of cocoons formed) on young cv. Tip Top in the laboratory (Table 2).

TABLE 2. Effect of a low dose (*c.* LC10) of neem seed kernel extract on *D.semiclausum* cocoon formation. Neem applied to Chinese cabbage leaf discs in a laboratory leaf-dip bioassay.

	Percent		
Treatment	*P.xylostella* larval mortality	*D.semiclausum* cocoons	*P.xylostella* adults + pupae
Control			
- *D.semiclausum*	6 a	-	94 b
+ *D.semiclausum*	43 b	23 a	34 a
Neem			
- *D.semiclausum*	10 a	-	90 b
+ *D.semiclausum*	34 b	47 b	19 a

Values within columns and followed by a common letter are not significantly ($P > 0.05$) different (ANOVA and pairwise LSD-tests, following arcsine transformation of percentage data).

Semi-field Trial

The neem treatments significantly ($P < 0.05$) reduced the number of larvae surviving to late fourth instar or pupation, although there were no significant ($P > 0.05$) differences in number of cocoons (*Diadegma* spp. or total) formed between neem-treated and control plants/ leaves. When total cocoon numbers were analysed as a proportion of the larvae which survived to the fourth instar, significantly ($P < 0.05$) more parasitoid cocoons formed on the treated plant material (82%) compared with the controls (69%). This effect occurred predominantly on the Sawi manis and Pak choy cultivars. Significant increases in parasitism with treatments compared with controls were not evident for *Diadegma* spp. alone, implying that cocoon enhancement occurred to a greater extent with *Cotesia* spp. A partial breakdown of the results is shown in Table 3.

DISCUSSION AND CONCLUSIONS

The resistance data presented support the notion that *P.xylostella* has the potential of overcoming virtually all attempts at chemical control, including strategies involving microbials (eg. *Bt)* and novel compounds such as abamectin. Of the three materials tested against the four field strains, only resistance to Florbac® (*Bt* ssp *aizawai*) was negligible. However, resistance to *Bt* ssp.*aizawai* has been demonstrated in other populations of *P.xylostella* (Tabashnik et al., 1994). Judicious use of such products is therefore imperative to prevent an escalation of resistance.

TABLE 3. Bionomic data based on recovered *P.xylostella* larvae from untreated and neem-treated plants after *c*. 3 days field-exposure to *Diadegma* spp. and *Cotesia* spp.

Treatment	Percent		Mean ± SE host larvae recovered / 4 plants (n = 5)
Host plant and cultivar	Parasitoid cocoons formed[1]	Parasitoid cocoons formed[1] / survivors[2]	
Control			
Tip Top	42 a	72 ab	118.5 ± 17.4 ab
Sawi manis	63 c	76 a	81.7 ± 9.8 ab
Pak choy	45 ab	60 b	26.9 ± 12.0 b
Neem[1]			
Tip Top	48 ab	79 a	125.6 ± 30.4 a
Sawi manis	56 bc	90 c	102.6 ± 19.7 ab
Pak choy	44 a	77 a	81.0 ± 17.5 ab

Values within columns and followed by a common letter are not significantly ($P > 0.05$) different (ANOVA and pairwise LSD-tests, following arcsine transformation of percentage data).

[1] Parasitism by *Diadegma* and *Cotesia spp.* only

[2] Larvae surviving either to pupation or succumbing to successful parasitism

The results of the tritrophic experiments show that plant intrinsic nutritional quality may have a significant effect on the third trophic level; plants of intermediate quality generally improving the rate of parasitoid cocoon formation compared with those of high or poor quality. *D.semiclausum* appeared to be more adversely affected by poor quality plants than *C.plutellae*, which is compatible with the greater susceptibility of the former species to a range of insecticides (Furlong et al., 1994). The phenomenon of parasitism enhancement by partially resistant plants is known to be robust, and has been demonstrated in other tritrophic laboratory studies with *P.xylostella*, including those where parasitoid oviposition of host larvae occurred in the absence of leaf discs (R.H.J. Verkerk, unpublished data). Such phenomena have been described for different species by Muldrew (1953), Salt (1956) and van den Bosch (1964). The use of a foliar-applied low dose of a neem seed kernel extract (*c*. LC10) was shown to enhance formation of *D.semiclausum* cocoons, presumably by inducing partial resistance in susceptible host plant cultivars. Reduction of the host's encapsulation rate on partially resistant compared with susceptible host plants, is the suggested mechanism, and further work testing this hypothesis is in progress. Some evidence of this effect , although less marked, was demonstrated in the semi-field trial. Whilst the method of induction of extrinsic host plant resistance requires further research, it is considered to have potential in integrated control strategies targeting *P.xylostella* and other agricultural pests known to develop high levels of resistance to a wide range of chemical groups.

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RESISTANCE OF LIGHT LEAF SPOT (*PYRENOPEZIZA BRASSICAE*) OF WINTER OILSEED RAPE TO MBC FUNGICIDES

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ABSTRACT

In 23 field trials carried out in Scotland between 1985 and 1994, MBC fungicides significantly reduced the levels of light leaf spot infection on winter oilseed rape in 5 out of 6 trials in the south-east of Scotland but in only 3 out of 17 trials in the north-east of Scotland. Preliminary studies into MBC resistance in the fungus *Pyrenopeziza brassicae* showed 25% of isolates obtained from 8 sites in north-east Scotland in 1994 were resistant.

INTRODUCTION

Light leaf spot (*Pyrenopeziza brassicae;* anamorph *Cylindrosporium concentricum*) is the most important disease of winter oilseed rape crops in Scotland, being favoured by long cool, wet winters. The disease first appears in the late autumn and reaches maximum levels in the spring, in March or April. Work carried out by the Scottish Agricultural College has shown that fungicide application in the autumn, even when little or no disease is present, gives good yield responses (Wale *et al.*, 1990), and growers routinely spray crops with fungicides in the autumn to control this disease. MBC generating fungicides have commonly been used at this time (Snowden *et al.*, 1991; Bowen *et al.*, 1993) because of their apparent efficacy and low cost. However, in the past three to four seasons, growers in Scotland have been increasingly dissatisfied with the control of light leaf spot achieved by MBC fungicides.

This apparent failure to control light leaf spot has led to the widely held view that resistance to MBC fungicides had developed in the light leaf spot fungus. This paper reports on the results of field trials carried out over a ten year period looking at the efficacy of MBC fungicides to control light leaf spot in winter oilseed rape, to determine whether there is evidence for the lack of effectiveness. In addition preliminary studies into possible MBC resistance in *Pyrenopeziza brassicae* are reported.

MATERIALS AND METHODS

Field trials

Field trials were carried out at 23 sites in Scotland from 1984/85 to 1993/94. Plots were drilled or burned out of field crops to give a minimum harvest plot size of 40 m^2. Trials

were of a randomised block design, with three or four replicates. Except for fungicide application, plots were treated as normal farm practice. Fungicides were applied using hand held or tractor mounted sprayers delivering 150-250 litres/ha. Untreated plots received no fungicide. All other plots received fungicides in the autumn (some also in early spring) and at stem extension, GS 3.5 (Sylvester-Bradley, 1985). At individual sites plots differed only in the fungicides applied in the autumn. MBC treated plots received 500 g AI/ha benomyl, carbendazim or thiophanate-methyl (as various products). Prochloraz treated plots received 250 g prochloraz/ha (as Sportak, 400 g/l EC; Sportak 45, 450 g/l EC; Sportak Alpha, 267 g/l SC), except at sites in south-east Scotland in 1988 and 1992 where 200 g AI/ha and 500 g AI/ha were applied. Disease assessments were carried out throughout the season but only those done when disease was at its maximum (mostly March and April) are shown. Yields were determined but are not reported in this paper.

MBC resistance tests

Plants were collected from 8 fields in the north-east of Scotland during the spring of 1994. Roots of the plants were wrapped in damp tissue and the plants incubated in a sealed polythene bag at room temperature for 24 to 48 hours to allow sporulation. Individual spore droplets (acervuli) were picked from leaves using a sterile syringe needle and placed onto V8 agar in petri-dishes. Isolates were allowed to grow for several weeks then sub-cultured onto fresh V8 agar to ensure pure cultures. Once the pure cultures reached a suitable size (5 cm diameter approximately), 5 mm plugs were removed from the leading edge of each isolate using a sterile cork borer and placed onto 4% potato dextrose agar containing 0, 1, 10, 100 or 2050 ppm carbendazim (the highest rate was estimated to be equivalent to field strength). Plates were incubated at $18^{o}C$ for four weeks then colony size was measured.

RESULTS

Field Trials

Table 1 shows the control of light leaf spot on winter oilseed rape at sites in north-east Scotland. In 12 of the 17 trials the average percentage leaf area infected (LAI) with light leaf spot in the untreated plots in the spring was low (less than 10%). In 5 of the 17 trials (1987,1990-91, 1993-94) the mean leaf area infected with light leaf spot was high (more than 10%). Where leaf area infected with light leaf spot in the untreated plots was low, the use of MBC fungicides in the autumn reduced infection by 0.4% LAI, which was an 18% control relative to the untreated. In only 2 out of the 12 trials in this category was reduction of disease by MBCs significant.

In trials where leaf area infected with light leaf spot in untreated plots was high, autumn applied MBC fungicides reduced infection in the spring by 0.6% LAI which was a 16.1% control relative to the untreated. In only 1 out of 5 trials was this reduction significant.

Similarly, the use of prochloraz based fungicides in the autumn in low disease pressure years reduced the leaf area infected in the spring by 0.1% compared to that of the untreated. The percentage relative control compared to the untreated was 27.2%. In only 2 of the 12 trials was this reduction significant. However, in high disease pressure years, the use of

TABLE 1. Effect of autumn applied MBC fungicides on levels of light leaf spot in winter oilseed rape in the spring, north-east Scotland 1985-94.

	% leaf area infected		% control relative to untreated	
	Low LLS year[a]	High LLS year[b]	Low LLS year[a]	High LLS year[b]
untreated	4.5	26.2	0	0
MBC	4.1	25.6	18.0	16.1
prochloraz	4.4	8.0	27.2	67.9
Total No. of trials	12	5		
No. trials where :				
MBC reduction significant	2	1		
prochloraz reduction significant	2	4		

[a] Low light leaf spot = less than 10% LAI
[b] High light leaf spot = greater than 10% LAI (1987, 1990-91, 1993-94)

TABLE 2. Effect of autumn applied MBC fungicides on levels of light leaf spot in winter oilseed rape in the spring, south-east Scotland 1988-93

	% leaf area infected			% control relative to untreated		
	Low LLS year[a]	High LLS year[b]		Low LLS year[a]	High LLS year[b]	
		all years	1993 only		all years	1993 only
untreated	9.3	26.5	42	0	0	0
MBC	0.5	10.6	44	94.6	70.7	0
prochloraz	0.6	3.7	11.8	93.5	87.9	71.9
Total No. trials	1	5	1			
No. trials where:						
MBC reduction significant	1	4	0			
prochloraz reduction significant	1	5	1			

[a] Low Light leaf spot = less than 10% (1988)
[b] High Light leaf spot = greater than 10%

prochloraz in the autumn significantly reduced the leaf area infected with light leaf spot in the spring by 18.2%, with a mean value of 67.9% control.

At only one of the six sites in south-east Scotland was leaf area infected with light leaf spot in the spring in untreated plots less than 10% (Table 2). At this site, the use of an MBC fungicide in the autumn significantly reduced leaf area infection in the spring to negligible levels or equivalent to 94.6% control. The use of prochloraz gave similar control.

At five of the six sites in south-east Scotland, light leaf spot infection in the untreated plots was high (greater than 10%). In four out of these five trials the use of an MBC fungicide in the autumn significantly reduced light leaf spot infection in the spring, reducing it from 26.5% in the untreated to 10.6% in the MBC treated plots (equivalent to 70.7% control). However, in 1993, when disease pressure was very high (42% LAI in the untreated in the spring), MBC fungicides failed to control light leaf spot. The use of prochloraz in this year gave 71.9% control of light leaf spot in the spring.

Results from field trials indicated that the use of MBC fungicides in the autumn in the north-east of Scotland did not reduce the over-wintered inoculum levels of the light leaf spot fungus *P. brassicae*, especially when disease pressure was high. In the south-east of Scotland the use of autumn applied MBC fungicides gave good control of light leaf spot, even in high disease pressure years, until 1993 when the fungicide failed.

MBC resistance tests

Seventy six isolates were obtained from 8 sites (Table 3). The isolates were very slow growing and after four weeks incubation at 18°C had reached an average diameter of 17.5 - 26.0 mm. 96% of isolates grew at 1 ppm carbendazim, with 25% of all isolates growing at 10 - 2050 ppm. However, individual sites differed in the number of isolates growing at each of the carbendazim concentrations. Of the seven isolates obtained from the site in Morayshire, 100% grew at 1 ppm carbendazim but all were controlled at 10 ppm. Similarly with isolates obtained from site number 2 in Gordon District and in Kincardine, all grew at 1 ppm carbendazim but were controlled at 10 ppm.

At the other five sites most of the isolates grew at 1 ppm carbendazim and were controlled at 10 ppm, but varying numbers grew at 10 - 2050 ppm. At site number 3 in Gordon District only one of the thirteen isolates (7.7%) grew at the higher concentrations but at site number 1 in Gordon, 25% of isolates grew at the highest concentration. 50% of isolates obtained from the Banff site grew at 2050 ppm. Of the isolates obtained from the two fields at the Angus site, 38.3% and 63.8% of isolates grew at the highest concentration of carbendazim.

Results showed that most isolates were inhibited at carbendazim concentrations of between 1 and 10 ppm. Nearly a quarter (23.7%) of isolates grown had an ED_{50} (effective dose of carbendazim giving 50% inhibition of growth compared with untreated control) less than 1 ppm carbendazim (Table 4), and approximately half of the isolates had an ED_{50} between 1 and 10 ppm carbendazim. A quarter (25%) of isolates had an ED_{50} greater than 2050 ppm carbendazim. Where isolates were not inhibited by carbendazim at 10 ppm they were also not inhibited by the much higher concentration of 2050 ppm.

TABLE 3. Effect of carbendazim concentration on the growth of *Pyrenopeziza brassicae* in vitro.

District	no. isolates	% isolates growing at each concentration of carbendazim (ppm)				
		0	1	10	100	2050
Morayshire	7	100	100	0	0	0
Banff	6	100	100	50	50	50
Gordon 1	12	100	91.6	25	25	25
Gordon 2	9	100	100	0	0	0
Gordon 3	13	100	100	7.7	7.7	7.7
Kincardine	5	100	100	0	0	0
Angus 1	11	100	81.8	63.6	63.6	63.6
Angus 2	13	100	100	38.3	38.3	38.3
Total	76	76	73	19	19	19
% of total		100	96	25	25	25

Each location corresponds to one field from one farm except in Angus where both fields were on the same farm.

TABLE 4. ED_{50} of isolates of *Pyrenopeziza brassicae* collected from eight sites in north-east Scotland, 1994

	concentration of carbendazim (ppm) giving 50 % inhibition of growth				
	< 1	1 - 10	10 - 100	100 - 2050	> 2050
Number of isolates	18	39	0	0	19
%	23.7	51.3	0	0	25

DISCUSSION

The use of prophylactic fungicide treatments in the autumn for the control of light leaf spot in winter oilseed rape is standard practice in Scotland. MBC fungicides are commonly used (Bowen *et al.*, 1993; Snowden *et al.*, 1990) although they are not recommended for use in second year rape crops where disease pressure is generally higher than that of first year rape crops. Until recently, growers were satisfied with the control achieved by MBCs. In the last three to four seasons control has been unsatisfactory and growers, particularly in the north-east of Scotland, have become increasingly concerned.

Trials reported here show that for most seasons during the mid to late 1980s disease pressure from light leaf spot was low and autumn applied fungicides did not reduce levels of infection compared to the untreated. Thus, although fungicides were being applied to field crops in the autumn, either MBC or prochloraz based, growers were assuming the low levels of disease were the result of fungicide activity. However, since 1990 disease pressure from light leaf spot has been high. MBC fungicides have been put under great pressure and have failed to control the disease in north-east Scotland. It is only because high levels of infection have occurred in crops in the spring during these last four seasons that growers have become anxious about the apparent failure of MBC to control light leaf spot. Prochloraz based fungicides still appear to be effective in controlling light leaf spot. In comparison, MBC fungicides were still working under high disease pressure in the south-east of Scotland until harvest 1992. In1993 MBC fungicides failed to control light leaf spot in this region.

In an investigation of sensitivity of *P. brassicae* to fungicides *in vitro*, Ilott *et al.* (1987) found field isolates were sensitive to benomyl and had an ED_{50} of less than 0.1 ppm. They did not find any natural resistant isolates. Only one out of eleven resistant mutant isolates, produced by u.v. irradiation in the laboratory, grew on 1 ppm benomyl. In later work Ball *et al.* (1990) found all 254 field isolates from East Anglia were sensitive to benomyl. Both groups of workers suggested if resistant isolates occurred naturally they must occur at very low frequency, too low to be detected by the sampling procedures. The work reported here showed that 96% of field isolates tested grew on 1 ppm carbendazim, with 25% having an ED_{50} greater than 2000 ppm carbendazim.

Resistance to carbendazim is present in the light leaf spot population in the north-east of Scotland but recent failure of MBCs to control light leaf spot in south-east Scotland suggests there may have been a recent shift in the population, from sensitive to resistant . Further investigation are needed to determine whether resistance is present in the south-east of Scotland.

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FUNGICIDE RESISTANCE CAN SPREAD IN *RHYNCHOSPORIUM SECALIS* POPULATIONS BY ASEXUAL RECOMBINATION

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ABSTRACT

Resistance to azole and benzimidazole fungicides has caused some practical disease control problems in *Rhynchosporium secalis*, the cause of barley leaf blotch. This threatened the effectiveness of strategies which use mixtures of these two fungicide groups to combat resistance. Spread of azole-resistance has been rapid, despite the fact that *R. secalis* is thought to move only short distances by rain splash, and lacks a sexual stage where recombination could occur. Resistance to benzimidazole fungicides has spread more slowly. Both azole and benzimidazole resistance occurred in several different genetic backgrounds indicating that spread is not clonal. We have now shown that pairwise mixtures, in culture, generated individuals with novel phenotypes, including one resistant to both fungicides. Novel isozyme patterns were generated, but did not correlate with changes in fungicide resistance. Where mixed infections occur in blotch lesions fungicide resistance may spread through natural populations of *R. secalis* by asexual recombination.

INTRODUCTION

Rhynchosporium secalis (Oudem) Davis, causes a damaging leaf blotch (or scald) disease of barley in cool, maritime regions worldwide. Control depends largely on the deployment of resistant barley cultivars, or use of fungicides, especially benzimidazoles and triazoles. Both these approaches have exposed considerable diversity within *R. secalis* populations, allowing selection of strains able to combat certain triazole fungicides (Kendall *et al*., 1993). Somewhat surprisingly, selection for benzimidazole-resistance has occurred only slowly, and considerable geographic variation exists in the frequency of benzimidazole-resistant strains (Taggart *et al*., 1994; Phillips & Locke, 1994).

Several studies involving pathogenicity tests (Zhang *et al*., 1992), isozyme (Newman & Owen, 1985; Goodwin *et al*., 1993) and RAPD (Whisson & Herdima, 1993) analysis have demonstrated that novel phenotypes can be generated simply from mixed *R. secalis* cultures either *in vitro*, or on plants. This feature of asexual recombination may be important for maintaining diversity within populations, since *R. secalis* has no known sexual stage (Goodwin *et al*., 1993). In this paper we explore the possibility that novel

fungicide resistance combinations might also be generated from mixed cultures of *R. secalis*, and the impact this might have on the spread of resistance within field populations.

MATERIALS AND METHODS

In vitro mixture experiments

Two pairs of isolates were chosen on the basis of different isozyme patterns and fungicide sensitivity. To obtain spore suspensions, plates were grown on Czapek-Dox + Mycological peptone (CDM, Kendall *et al.*, 1993) for 14 days at 17°C, then flooded with sterile distilled water and the surface scraped with a glass rod. Spore suspensions were adjusted to 2 x 10^5/ml. The isolate pairs (554.24 x 765.03.01; 810.39.02 x 811.36.01) were mixed thoroughly, and 1 ml of the mixture spread on CDM plates. After 20 days incubation, single spore isolates were obtained, tested for the sensitivity to carbendazim, diethofencarb, and triadimenol, and their isozyme patterns analysed.

Mixture experiments on plants

Seeds of a susceptible barley cultivar (Tyne) were grown and inoculated as described elsewhere (Kendall *et al.*, 1994) with a 1:1 mixture of the two isolates 810.39.02 and 811.36.01. Lesions were excised from infected leaves, and *R. secalis* isolated (Kendall *et al.*, 1993) and tested for fungicide sensitivity and isozyme analysis.

Fungicide sensitivity assays and isozyme analysis

Fungicides (all technical grade) were added to CDM before autoclaving (120°C; 15 min) at concentrations which would allow determination of Minimum Inhibitory Concentrations, (MIC). Medium (2 ml) was pipetted into each of the 25 wells in "Repli-Plates" (Sterilin, Stone, UK). Wells were inoculated with a 100 µl suspensions containing 1 x 10^4 spores/ml, incubated at 17°C for 14 days, and growth assessed to establish the MIC. Standard strains of known sensitivity were included in all assays.

Cells were grown in liquid MYMPG (Kendall *et al.*, 1994) medium for 12 days at 17°C with constant shaking (120 rev/min). Cells were collected by filtration, washed with distilled water, and stored frozen (-20°C) until used. Cells (2 g) were ground to a fine powder in liquid nitrogen in a mortar with acid washed sand, and extracted with buffer (5mM Tris HCl, pH 7.0; 2% (wt/*V*) Triton X-100 and 15% (w/v) sucrose) for 60 min at 4°C. Extracts (1.5 ml) were transferred to Eppendorf tubes and clarified by centrifugation at 13,000 rpm for 5 min. Pellets were resuspended in buffer (1.0 ml), centrifuged again, and the combined supernatants stored at -80°C. Isozymes were separated on 6.5% polyacrylamide gels using a horizontal electrophoresis apparatus (Biorad, Hemel Hempstead, UK) according to standard conditions described by Kendall (1994). Wells were loaded with 10 µl of each extract, and Bromophenol Blue used to identify the electrophoresis front. α-Esterase, catalase and phosphoglucomutase (PGM) were stained according to established procedures (Kendall, 1994).

RESULTS

Characteristics of parental isolates

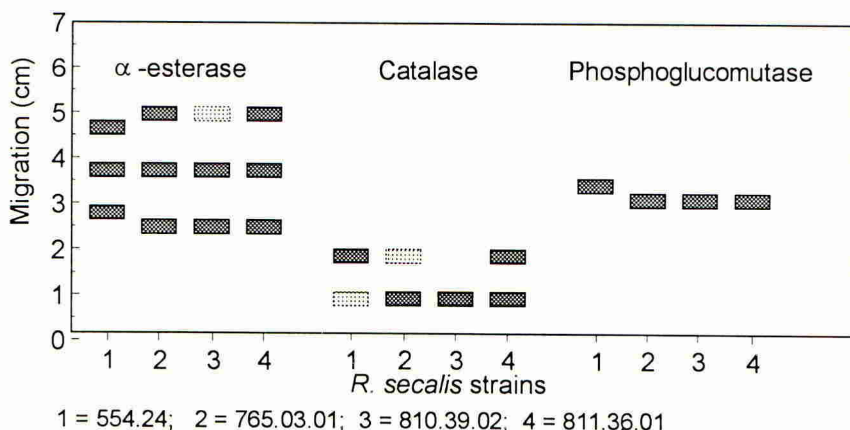
Four *R. secalis* strains were selected which differed in their sensitivity to fungicides (Table 1) and isozyme patterns (Figure 1). As expected, strains showed negative cross resistance between carbendazim and diethofencarb, but no link between carbendazim and triadimenol sensitivity. Carbendazim resistance reflected the substitution of glycine at amino acid position 198 in the target ß-tubulin, for glutamic acid in the sensitive strain (Wheeler *et al.*, 1994). Isozyme analysis showed polymorphism in all three enzymes, α-esterase, catalase and PGM, which allowed pairwise mixtures to be established on CDM involving clearly marked phenotypes.

TABLE 1. Fungicide sensitivity of four strains of *Rhynchosporium secalis* used in mixture experiments.

Strain	Benzimidazole (H)	Diethofencarb (D)	Triadimenol (T)	Amino acid at codon 198 in ß-tubulin
554.24	S	R	S	Glutamic acid
765.03.01	R	S	R	Glycine
810.39.02	S	R	R	Glutamic acid
811.36.01	R	S	S	Glycine

S = Sensitive; R = Resistant (MICs for carbendazim > 100 μg ml^{-1}, for diethofencarb > 100 μg ml^{-1} and triadimenol > 10 μg ml^{-1} were considered as resistant)

FIGURE 1. Isozyme patterns of four *Rhynchosporium secalis* strains used in crossing experiments.



Stability of parental isolates

Fifty daughter clones were isolated from each of the four parental strains, and tested for their fungicide sensitivity. All these daughter clones were identical to their respective parents. Isozyme analysis also confirmed the stability of the banding patterns for α-esterase, catalase, and PGM.

Analysis of single spore isolates recovered from mixed cultures

Three hundred single spore isolates were recovered from the mixture of 554.24 and 765.03.01 after 20 days incubation. Three of these isolates were unlike the "parents" and showed novel fungicide sensitivities when first assayed (Table 2), and altered isozyme patterns. However, the two isolates resistant to all three fungicides were not stable, and on repeated subculturing reverted to carbendazim sensitivity and diethofencarb resistance, but remained triadimenol resistant. Sequencing of one of these unstable isolates confirmed the presence of glutamic acid at codon 198. In addition, three other isolates which resembled 554.24 in their fungicide sensitivities had altered isozyme patterns with an additional, faster migrating, catalase band not found in either "parent".

143 single spore isolates were recovered from the second mixture between 810.39.02 and 811.36.01, and nine of these represented a novel phenotype resistant to all three fungicides (Table 2). The stability of these isolates and their DNA sequence around codon 198 is currently being examined. However, all nine isolates had isozyme patterns identical to 810.39.02. Six isolates were recovered with an additional catalase band but in all other respects resembled 811.36.01.

Unfortunately, *R. secalis* strains 554.24 and 765.03.01 were no longer pathogenic and only a mixed infection of 810.39.02 and 811.36.01 was successful on plants. So far, 136 isolates have been recovered and all but four resembled 810.39.02. Four isolates, however, were resistant to all three fungicides. The stability, isozyme patterns, and DNA sequence of the ß-tubulin gene fragment surrounding codon 198, are currently being determined for these isolates.

TABLE 2. Fungicide sensitivity of isolates recovered from mixed infections of *Rhynchosporium secalis in vitro*.

Mixture	No. isolates tested	Progeny Phenotype							
		HS DR TS	HR DS TR	HS DR TR	HR DS TS	HR DR TR	HS DS TS	HR DR TS	HS DR TS
554.24 x 765.03.01	300	40	257	1	0	(2)	0	0	0
810.39.02 x 811.36.01	143	0	0	90	44	9	0	0	0

Isolates in parenthesis were not stable

DISCUSSION

Lack of a known sexual stage precludes formal genetic analysis of fungicide resistance in *R. secalis*. However, molecular analysis of benzimidazole resistance has identified a point mutation on the nuclear ß-tubulin gene linked to resistance (Wheeler *et al*., 1994), but the genetic control of azole resistance has not been explored in this way. Unlike the situation normally found for DMI fungicides, selection of *R. secalis* in the field generated a distinct triadimenol resistant population (Kendall *et al*., 1993), suggesting that resistance to this azole fungicide might be controlled by one gene. It is difficult to distinguish between recombination events and mutations in mixture experiments, but changes in both fungicide sensitivity and isozyme patterns, are unlikely to arise through mutation. For changes in diethofencarb and carbendazim sensitivity, DNA sequence analysis of the ß-tubulin gene should help identify mutations.

Regardless of the genetic basis of azole resistance, novel fungicide resistance phenotypes have been recovered from mixed infections both *in vitro* and *in vivo*. Not all these new phenotypes were stable and may not be recombinants, but three isolates from the 554.24 x 765.03.01 mixture seem to have arisen by recombination. Asexual recombination offers a way to explore the genetic control of azole resistance in *R. secalis* using the parasexual cycle (Pontecorvo & Roper, 1952), and isozyme and RAPD characters as additional markers. The three enzymes used in this work provide examples of isozyme polymorphisms, and confirm that mixed infections generate novel isozyme patterns (Newman and Owen, 1985), although this does depend on the *R. secalis* strains chosen as mixture partners.

The practical significance of asexual recombination arises from increased gene flow it generates within otherwise clonal pathogen populations. *R. secalis* conidia are thought to be dispersed by rain splash and, consequently, do not migrate over large distances. During epidemics *R. secalis* populations are large, and probably all possible mutations occur at some time. Asexual recombination counteracts the effects of selection and genetic drift on these mutations, and maintains genetic diversity. Selection for fungicide resistance will be slowed, especially where a resistance allele carries a fitness penalty. This may explain why certain mutations conferring carbendazim resistance, and associated with poor pathogenicity (Kendall *et al*., 1994) have not been recovered from field populations (Wheeler *et al*., 1994), even though benzimidazole fungicides have been used on barley crops for many years. The fitness penalty apparently associated with triadimenol resistance was much less (Kendall *et al*., 1993) and resistance to this fungicide has spread rapidly, even into populations not intensively treated with azole fungicides.

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SHIFTS IN MORPHOLINE SENSITIVITY OF THE WHEAT POWDERY MILDEW PATHOGEN, ERYSIPHE GRAMINIS F.SP. TRITICI, AND THEIR INFLUENCE ON DISEASE CONTROL

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ABSTRACT

A survey has been undertaken since 1987 to investigate fenpropimorph sensitivity of *Erysiphe graminis* f. sp. *tritici* in different European regions. Results obtained from airborne samples show that remarkable regional differences exist. First shifts towards a decreased sensitivity were observed in 1989 in Northern Germany and Denmark, whereas in other areas the shifts occured later. However, up to the present the mean resistance factor has not exceeded a factor of 10x in any area surveyed.

Cross-sensitivity relationships were determined for field isolates from different countries. There was evidence to suggest a positive correlation between sensitivities to fenpropimorph and fenpropidin for selected wheat powdery mildew isolates.

In areas of reduced sensitivity, performance of both compounds remains unchanged. Data from two field trials showed high levels of efficacy for fenpropimorph and fenpropidin against wheat powdery mildew. However, for fenpropimorph, the safety margin for both rates and application timing, can be reduced in regions where a shift has occurred. In these cases it is important to follow strictly the use recommendations in order to obtain good control under unfavourable conditions.

INTRODUCTION

Wheat powdery mildew, caused by *Erysiphe graminis* f.sp. *tritici*, is frequently epidemic in North-west Europe, and is therefore one of the main target pathogens for chemical disease control. Over the last decade, most treatments have been based on two groups of active ingredients, namely azoles (demethylation inhibitors: DMIs) and morpholines/morpholine-like-compounds. Both inhibit sterol biosynthesis of the fungus, but have different modes of action. During the 1980s, it became evident that there was a partial loss of DMI sensitivity by the pathogen. Today, much data is available on sensitivity as well as on cross-resistance relationships in DMI fungicides, whereas there is still a lack of information about the ability of the wheat powdery mildew to adapt to morpholines.

To determine the sensitivity of wheat powdery mildew towards fenpropimorph and to study the changes in sensitivity of pathogen populations with time, a European-wide monitoring programme was started in 1987. A short report on the evolution and current status of fenpropimorph sensitivity of the pathogen on a European scale has been provided recently (Felsenstein, 1994b). Fenpropidin was included in the investigations since 1992. The present report shows data on sensitivity towards both compounds, fenpropimorph and fenpropidin, with regard to cross-sensitivity relationships. An assessment of sensitivity shifts on disease control is also given on the basis of field trials.

MATERIALS AND METHODS

Collection of the pathogen samples for analysis was carried out in two different ways. In one part of the study, random samples were taken from the air above areas of interest, so that reliable data from different regional mildew populations could be produced. Conidio-spores were collected with a jet spore trap (Schwarzbach, 1979) mounted on the roof of a car. At least ten isolates per region were analyzed for their sensitivity towards fenpropimorph (since 1987). A detailed description of the test procedure is given in Felsenstein (1991, 1994a).

In a second part, data was also obtained from random field samples collected in different countries to get information with respect to more local situations and specific trials. Fields were either treated or untreated and samples were taken during the whole season. Mildew-infected leaves were sent from different sites in Ireland, Denmark, UK, France and Germany by co-workers of Ciba. In the laboratory, conidio-spores of freshly-sporulating colonies were transferred onto leaf segments, and their sensitivities were assayed. Four single colony isolates were tested per sample for their sensitivity towards fenpropimorph. After the evaluation of LD50-values a smaller number of isolates, representing a wide range of sensitivities to fenpropimorph, was selected and also tested against fenpropidin to examine cross-sensitivity between the two compounds. Data from France and Germany is presented.

Standard (wild-type) isolates were included in the sensitivity tests. They were obtained from the field in the 1970s, before the fungicides in question were commercialized, and therefore represent the sensitivity of the fungus in original, unselected populations. If the sensitivity of each test isolate is related to that of the standard isolates, a resistance factor (RF) can be calculated. To characterize each random sample, the mean resistance factor (MRF), was determined.

Two field trials were carried out in 1992, one in UK and one in Germany, to examine the performance of fenpropimorph and fenpropidin against wheat powdery mildew. One early season treatment at the full rate (750 g AI/ha) and the half rate (375 g AI/ha) of both compounds was applied with 200 l/ha spraying volume just after first symptoms were visible. The percentage of infected leaf area was visually assessed at approximately weekly intervals in the treated plots and the untreated check plots, respectively.

RESULTS

Sensitivity situation

Figure 1 shows European-wide sensitivity data of the wheat powdery mildew towards fenpropimorph within a time scale from 1987 to 1993.

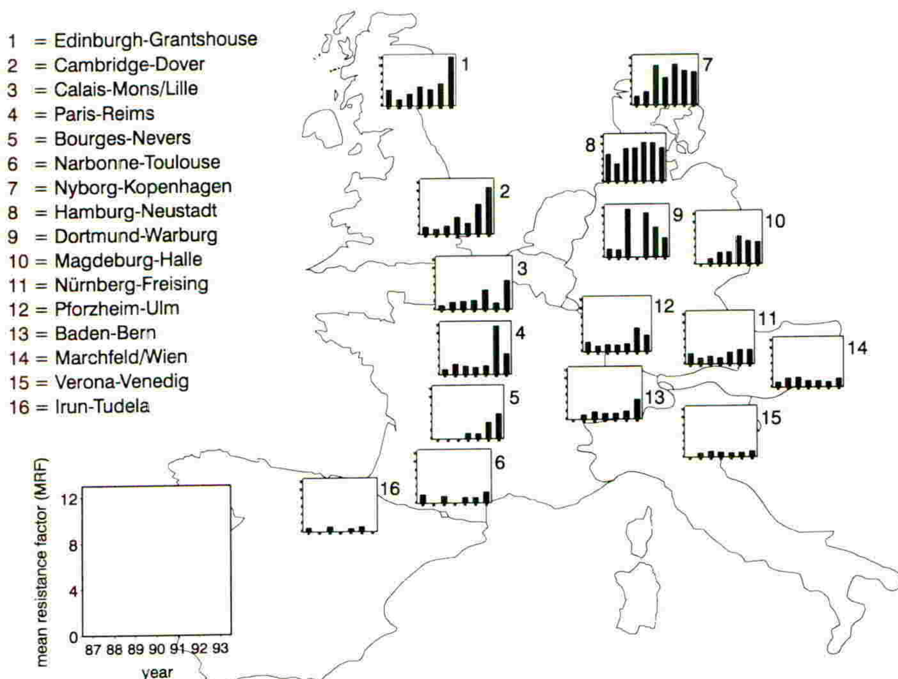

FIGURE 1. Mean resistance factors (MRFs) of random samples of the wheat mildew pathogen out of different European wheat growing areas towards fenpropimorph from 1987 to 1993.

According to 16 specific regional mildew populations, which are considered to represent the whole range of sensitivity of the pathogen within Europe, MRF-values from airborne random samples (jet spore trap) are presented for each year investigated (more data in Felsenstein, 1994b). Results show that, within Europe, there are remarkable regional differences in the current sensitivity situation of the wheat mildew towards fenpropimorph as well as differences in the population shifts.

At the start of the investigations in 1987, only two populations, one in Scotland and one in Northern Germany, had reached a markedly higher MRF with a value of ≥ 3, if a population with an MRF of less than 3 is considered to possess an unselected or nearly unchanged sensitivity level (Felsenstein, 1991). In the following years, more and more populations within North-west Europe exceeded an MRF of 3, and only populations in the South and East of Europe remained on their former sensitivity level, close to that of the standard isolates. However within the seven-year period of observation of European wheat mildew from 1987 to 1993, no regional population was able to reach a markedly higher MRF than 10. This even applies to the population in Northern Germany between Hamburg and Neustadt, where MRFs varied between c. 5 and 10 during the whole investigation period, despite the relatively high selection pressure.

The changes in the level of fenpropimorph sensitivity of the populations within North-west Europe can be well illustrated by the sensitivity structure of the samples. In Figure 2, five examples are given which represent the current spectrum of populations' structure within Europe and the different evolution stages of populations' sensitivity dynamic, respectively (1993 data). Five types of population structures have become apparent: There is 'Type 1' which is currently typical for the South of Europe beyond the Alps and Pyrenees. There is only little variation in fenpropimorph sensitivity and up to now, no isolate has been detected which reached an RF ≥ 3. 'Type 2' is characterized by a large part of isolates with an RF < 3 and a small part of isolates with an RF ≥ 3. This represents the current sensitivity situation of Southern France and Eastern Europe. The MRFs of such populations are, in general, still close to 1. Whereas in 1987, all analyzed populations within Europe, (except the two mentioned above), possessed a sensitivity structure of 'Type 1' and 'Type 2', respectively, the present situation is that the structures of most populations in North-west Europe are similar to those of 'Type 3' and 'Type 4'. Firstly, there is typically a wide range of LD50-values (or RFs) within the populations and secondly, a continuing decrease of the number of isolates with a wild-type sensitivity. In most cases, the MRF exceeds the limit of 3, and, because of the broad and relatively homogeneous range of sensitivity, an evident variation in the MRF-value from year to year has to be taken into account, until the population dynamic will reach the structure of 'Type 5'. There are only a few populations in the North of Europe, which have reached this composition. In comparison with 'Type 3' and 'Type 4', LD50-values within the sample are not so widely spread. Isolates with an unchanged or nearly unselected sensitivity are very rare or cannot be detected. The MRF of such populations varies around 10. At the present time, 'Type 5' is the most advanced stage of population dynamic due to the gradual loss of fenpropimorph sensitivity of the European wheat mildew pathogen.

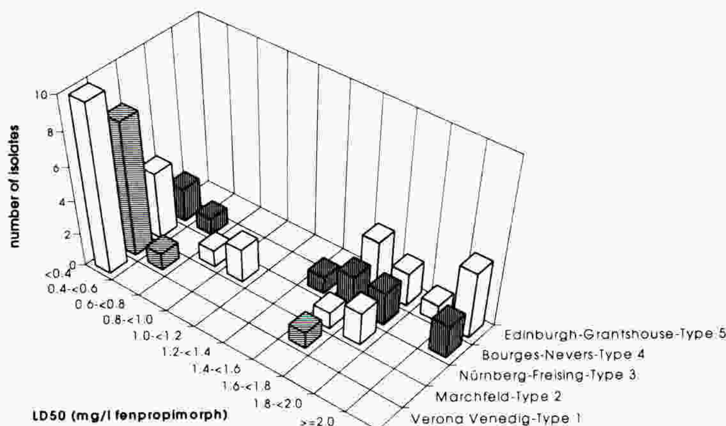

FIGURE 2. Sensitivity structure of 5 European wheat mildew populations, representing 5 different evolution stages within the population dynamic due to the gradual loss of fenpropimorph sensitivity.

Cross-sensitivity to fenpropidin

Results obtained for selected field samples from different sites in France and Germany are demonstrated in figure 3. Data correspond, in general, to those obtained from air-borne random samples. A wide range of sensitivities could be detected for the field samples towards fenpropimorph and fenpropidin in 1992 (data not shown) and 1993. Furthermore, it is obvious that there are some similarities in the sensitivity of the pathogen towards fenpropimorph and fenpropidin, although RF-values towards fenpropimorph in most cases tend to be higher in comparison to fenpropidin. The positive correlation between the sensitivities of the pathogen to the two compounds is also reflected in data from Germany, if it is considered that in this country fenpropidin has never been used in the agricultural practice, and thus no direct selection pressure has taken place up to the present.

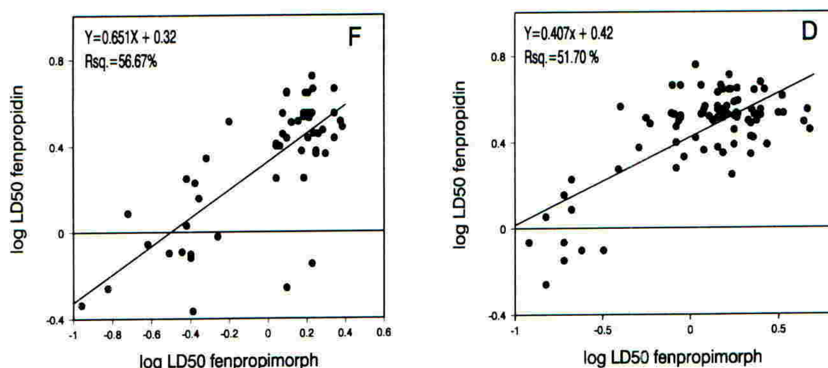

FIGURE 3. Cross-sensitivity of *E. graminis* f.sp. *tritici* between fenpropimorph and fenpropidin for selected field isolates collected in France (F) and Germany (D), 1993

Field performance

Table 1 summarizes performance results of fenpropimorph and fenpropidin against wheat powdery mildew from two trials carried out in 1992. Trials were located in one region in UK and Germany respectively, where a shift towards decreased sensitivity has occurred.

TABLE 1. Control of *E. graminis* f.sp. *tritici* on winter wheat (*cv. Apollo*, UK; *cv. Kanzler*, D) in field trials in UK and Germany 1992 (1 application per trial)

country	compound	g a.i./ha	% infected leaf area			
			6 daa	13 daa	24 daa	31 daa
UK	check		14	24	25	51
	fenpropimorph	375	3	8	13	23
	fenpropimorph	750	2	4	7	9
	fenpropidin	375	2	5	5	9
	fenpropidin	750	2	2	2	2
	LSD 0.05		1.35	1.79	3.83	9.73
			5 daa	13 daa	21 daa	29 daa
D	check		11	12	11	33
	fenpropimorph	375	9	8	7	19
	fenpropimorph	750	7	4	2	5
	fenpropidin	375	6	4	3	5
	fenpropidin	750	7	3	1	2
	LSD 0.05		9.21	8.75	7.37	10.78

daa = days after application
trial in D = curative treatment with 3-4 % infected leaf area and in UK 2-10% at application date

No significant differences could be detected in the level of attack for the full rate plots of both fungicides and the half rate of fenpropidin. In both trials however, the full rate of fenpropidin gave the best powdery mildew control. For the half rate of fenpropimorph it can be seen that powdery mildew control was significantly lower in comparison to the other treatments under the given conditions. In addition, the trial in UK with high disease pressure revealed a better initial activity and a longer lasting effect of fenpropidin in a rate by rate comparison of the full doses of the two fungicides, although differences were not statistically significant.

DISCUSSION

Results presented provide a clear survey on the recent evolution and current situation of the wheat mildew pathogen towards fenpropimorph on an European scale. It has to be emphasized that the fungus possesses a measurable ability for sensitivity adaptation. Current data reveals regional differences in morpholine sensitivity within Europe, with a spectrum of MRFs from 1 (= no reduced sensitivity) to ~10. Since 1987, first MRF-shifts were observed in Scotland, Northern Germany and Denmark. In other areas of North-west Europe the shifts occurred later. The MRFs remained on their former level only in Eastern and Southern Europe. Thus, as MRFs $\geq$ 3 have been obtained from areas with a relatively frequent or extensive use of appropriate fungicides, it is postulated that the loss of sensitivity mainly depends on selection pressure. In addition, wind dissemination of the pathogen seems to have an increasing influence on the sensitivity of some regional wheat mildew, by mixing of neighbouring populations. Especially in the recent past, a variation in MRF-values from year to year has been obtained for some populations in North-western Europe. This observation is very characteristic for regional populations which have a wide range in their sensitivity and have reached a structure, as 'Type 3' and 'Type 4' described above. Up to the present, the wild-type population structure ('Type' 1) with a typical narrow sensitivity range has only been maintained in Southern Europe, where neither selection pressure nor wind dispersal of the pathogen had a measurable influence on sensitivity. Comparable observations had been obtained by Felsenstein (1991) during the 1980s in European-wide investigations on triadimenol- (DMI-) sensitivity of wheat powdery mildew.

Overall results show a relatively moderate population dynamic with regard to a loss of sensitivity to fenpropimorph during the last decade. In most populations of North-west Europe, the MRF-level first changed markedly at the beginning of the 1990s. Only population structure indicated dynamics at an earlier time (Felsenstein, 1991; Lorenz *et al.*, 1992). Furthermore, the mean resistance factor of the populations nowhere exceeded a level of about 10, independent of the time of the shifts. It is surprising that, despite a high selection pressure during the last 10 years, the sensitivity level of the population between Hamburg and Neustadt in Northern Germany has not changed markedly since at least 1987. This observation as well as data from the other populations indicates that an MRF of about 10 is a first serious limit with respect to a further loss in fenpropimorph sensitivity. The main reason for the moderate resistance dynamics and the slightly reduced sensitivities towards both compounds might be due to the fact that morpholines (and piperidines) are considered to act as two-site-inhibitors (Buchenauer, 1984; Kato, 1986; Kerkenaar, 1987). In addition, the polygenic control of resistance evolution towards morpholines combined with a markedly genetical recombination of the wheat powdery mildew fungus (Felsenstein, 1991) should be taken into account. The more resistant the pathogen becomes, the more genetic changes are necessary. Because of an intensive yearly redistribution of genes responsible for a loss in morpholine sensitivity, maintenance of pathotypes with an outstanding resistance factor is probably unimpeded.

Data shows that there is a positive cross-sensitivity of the pathogen towards the two compounds, fenpropimorph and fenpropidin. In contrast to a reduction of sensitivity caused by selection pressure, LD50-values towards fenpropidin from 0.71 to 5.71 mg/l obtained for field samples from different sites in Germany in 1993 can only be explained by a positive correlation in the sensitivity towards the two compounds. However, it has to be considered that the isolates tested were preselected to represent a wide range of fenpropimorph sensitivity and thus the correlation would have been smaller for randomly chosen isolates. At the same time it has to be stressed that the similarities are relatively vague and not very close, respectively. Comparable observations have been obtained by investigations on the cross-sensitivity of cereal mildew towards demethylation inhibiting fungicides (Gisi *et al.*, 1986; de Waard *et*

al., 1986; Enisz, 1988; Felsenstein, 1994a), where the pathogen reached different resistance levels towards different azole-compounds.

Performance data from the trials in UK and Germany show that at present the efficacy of both fenpropimorph and fenpropidin seems to be unaffected by the sensitivity shift. Similar experiences have been recently reviewed by Hollomon (1994). However, it becomes evident that in a situation of decreased sensitivity and high disease pressure the margin of errors for deviations from label recommendations can be reduced. In this respect, the data of the two field trials shows an advantage of fenpropidin over fenpropimorph with regard to a better initial efficacy and longer lasting effect for wheat powdery mildew control. Fenpropimorph still gives good control under these conditions if it is applied at the correct rate (750 g AI/ha) and timing. This shows that in areas of reduced sensitivity it is important to adhere to the use recommendations for the two compounds. In the same context a further aspect has to be critically mentioned, namely the splitting and/or the application of reduced rates of the compounds. In a recent publication Forster *et al.* (1994) demonstrate the negative effect of such practices with regard to a stronger selection process towards a decrease in sensitivity during a season, although performance levels were high. Therefore the recommendations for application should be strictly followed concerning both rate and intervals to prevent sensitivity shifts and to give good powdery mildew control.

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RAPD ANALYSIS OF 515 EYESPOT ISOLATES FROM FIELD SITES IN EUROPE

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ABSTRACT

Random amplification of polymorphic DNA (RAPD) by the polymerase chain reaction (PCR) was used to classify of 515 isolates of the cereal eyespot fungus *Pseudocercosporella herpotrichoides*, into the W– and R–pathotypes. Samples originated from selected field sites in Europe. Routine analyses of eyespot populations are based on the visual assessment of colony morphology on agar media, with W–types producing fast, even–edged colony growth (F/E) and R–type colonies exhibiting slower growth with feathery margins (S/F). The ratios of W– and R–types, as shown by the use of RAPD, were in line with those observed in previous years by other workers but the existence of fast–growing and/or even–edged R–types, normally undetectable by conventional methods, was revealed. Application of RAPD analysis for the classification of 515 eyespot field isolates clearly demonstrates how cultural methods fail to accurately discriminate the two pathotypes either on the basis of colony morphology or radial colony growth rate.

INTRODUCTION

Pseudocercosporella herpotrichoides (Fron) Deighton (perfect state, *Tapesia yallundae*) is the causal agent of eyespot disease of cereals. Pathotypes of this species are defined by their ability to infect a range of cereal hosts in conjunction with *in vitro* growth characteristics (Lange–de la Camp, 1966). Two major pathotypes of *P. herpotrichoides* have been identified in Europe; Wheat–types (W), that are more pathogenic to wheat than to barley and rye, and Rye–types (R), that are equally infectious to all three cereal hosts. The W– and R–type classification initially described by Lange–de la Camp, (1966) has subsequently been refined and confirmed by other workers (Scott *et al.*, 1975; Scott & Hollins, 1980; Hollins *et al.*, 1985). Methods currently in widespread use for the identification of W– and R– pathotypes include colony morphology on nutrient–rich agar medium (Hollins *et al.*, 1985) and pigmentation beneath the colony on maize meal agar (Creighton, 1989). In pure culture on nutrient–rich agar media such as potato–dextrose (PDA) or malt–yeast–glucose (MYG), W–types typically have even–edged colonies, whereas R–types produce uneven colonies with feathery margins that grow at about half the rate of W–types (Lange–de la Camp, 1966; Scott *et al.*, 1975; Papaikonomou, unpublished).

Identification of putative pathotypes in culture rather than by pathogenicity testing on cereal hosts has the advantage of speed and low cost but is complicated by the existence of strains with apparently intermediate morphology (Hollins *et al.*, 1985; Gallimore *et al.*, 1987; Creighton & Bateman, 1988; Creighton & Bateman, 1991). Further problems arise due to the phenotypic instability found in some R–type isolates, whereby part of an S/F colony sectors and acquires F/E growth, indistinguishable in culture from that of genuine W–types (Julian *et al.*, 1994).

An important change in eyespot populations has been the replacement over the last decade of benzimidazole–sensitive strains of the fungus by benzimidazole–resistant strains. Carbendazim resistant isolates were first collected from stubble in Germany in 1974 (Rashid & Schlösser, 1975) and are now widespread (Brown *et al.*, 1984; Griffin & King, 1985; King & Griffin, 1985; Hollins *et al.*, 1985; Hocart *et al.*, 1990). The imidazole prochloraz, which is widely used against eyespot, still offers adequate levels of control against both W– and R–

types in most countries where cereals are intensively grown. However, there have been some reports that resistance to this fungicide may be developing in France (Leroux *et al.*, 1988; Cavelier *et al.*, 1992).

Reliable and accurate discrimination of W– and R– pathotypes is a prerequisite for the study of the population biology and epidemiology of the fungus and for analysis of the effects of environmental factors such as temperature and rainfall. Since a differential response of W– and R– pathotypes to many commercial fungicides is well known (Leroux *et al.*, 1988), responses of field populations of *P. herpotrichoides* to different fungicides and spray regimes also requires accurate identification of pathotypes.

Surveys to monitor population shifts of W– and R–types throughout Europe, in particular those related to modified sensitivity to fungicides, require accurate processing of large numbers of samples. For this reason there has been a reliance on cultural characters to classify isolates. Recent advances in molecular fingerprinting technology have indicated that cultural methods for W– and R–type identification may be unreliable. DNA polymorphisms have been shown to give unequivocal discrimination of pathotypes (Nicholson & Rezanoor, 1994).

Randomly amplified polymorphic DNA (RAPD) analysis relies on the polymerase chain reaction (PCR) to generate simple and reproducible fingerprints of complex genomes. This procedure has the advantages of being technically simple and quick to perform, requires small amounts of DNA, and does not use radioactivity. RAPDs are well suited for large sample–throughput systems and are thus ideal for studies of biodiversity, such as in the *P. herpotrichoides* pathosystem. In the current study, the technique was used to analyse 515 field isolates of the fungus and comparisons were made with cultural morphology and growth rate.

MATERIALS AND METHODS

Origin of Field Isolates

The isolates analysed in this project were from field sites in France, Germany and the UK. The strains from France were part of a survey carried out by AgrEvo UK Limited in 1993, undertaken to monitor temporal shifts in the relative proportions of W– and R–types, as well as genetic variation in field isolates.

Strain Purification

Purity of isolates was ensured by transfer of colony hyphal tips onto malt– yeast extract and glucose agar (MYG; Julian & Lucas, 1990) amended with penicillin (50 μg/ml) and streptomycin sulphate (100 μg/ml) to inhibit growth of bacterial contaminants. Single sporing of isolates was used in some cases to ensure genetic uniformity. Conidia were produced on water agar (TWA; agar, 20 g/l dissolved in tap water). Cultures were incubated at 10°C for 2–4 weeks to yield sufficient mycelium for assessment of colony morphology and growth rate assays.

Colony Growth Rates and Morphology

Isolates of *Pseudocercosporella herpotrichoides* were assessed for growth rate and colony morphology on MYG. A total of 9 replicate colonies per strain were grown from 2.5 mm agar plug inocula on MYG plates at 19°C in the light, for two weeks before colony diameters were measured and used to calculate radial growth rate (mm/day). Colony morphology was scored as even (E), feathery (F) or intermediate (INT) for those isolates that could not be clearly categorised into the first two groups. Cultures were incubated under the same conditions for another 2–4 weeks prior to DNA extraction for molecular fingerprinting.

DNA Extraction and RAPD Analysis

Randomly amplified polymorphic DNA (RAPD) markers (Williams *et al.*, 1990) were used to differentiate isolates of *P. herpotrichoides*. Mycelium (area 2–3 cm^2), scraped off 4–6 week old cultures grown on MYG, was suspended in 0.6 ml TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and boiled for 5 min. Samples were then cooled on ice for 5 min and 0.6 ml of a *V/V* 25:24:1 mixture of TE saturated phenol : chloroform : isoamyl alcohol added. The resulting suspensions were vortex mixed and centrifuged (11,600g, 5 min) and the supernatant removed. An equal volume of chloroform : isoamyl alcohol (24:1) was added to the new solution which was then vortex mixed and centrifuged (11,600g, 5 min) and the supernatant removed and stored at 4°C.

RAPD analysis was subsequently performed as previously described by other workers (Williams *et al.*, 1990; Nicholson & Rezanoor, 1994). Aliquots of the supernatant (1 µl) were added to 49 µl reaction volumes containing 100 µmol each of dATP, dCTP, dGTP and dTTP (Pharmacia), 0.5 µl each of a Tween 20 (0.05 ml/ml) and Nonidet P–40 (0.05 ml/ml) solution (Sigma), 10 pmoles of a single 10–base primer, 5µl of a 10–fold PCR reaction buffer and 1 unit of Taq DNA polymerase (Boehringer Mannheim). Primers OPA–10 (GGGTAACGCC), OPB–04 (GGACTGGAGT) and OPB–11 (GTAGACCCGT) (Operon Technologies, USA) were used to screen the eyespot isolates.

Genomic DNA was amplified using a Perkin–Elmer 480 thermocycler for the polymerase chain reaction, with 45 cycles of 94°C for 15s (ramp rate 30°C min^{-1}), 36°C for 1 min (ramp rate 30°C/min) and 72°C for 1 min (ramp rate 60°C/min) before a final elongation stage of 5 min at 72°C. Amplification products were separated by electrophoresis in 1.5% agarose gels and visualised by staining with ethidium bromide.

Additional DNA Cleaning Procedures

Where solutions of isolate genomic DNA (often from old cultures) gave poor or no amplification, equal volumes of CTAB buffer (0.2M Tris–HCl, 0.05M EDTA, 2.0M HCl and 2% CTAB) were added to 200µl of those and incubated at 65°C for 15 min, before 400µl chloroform : isoamyl alcohol (24:1) was added. The resulting mixture was vortex mixed and centrifuged at 11,600g for 5 min. DNA was precipitated from the supernatant by addition of an equal volume of isopropanol, incubation of the solution at -20°C overnight and centrifugation at 11,600g for 5 min. The resulting pellet was vacuum–dried, resuspended in 50µl TE buffer and stored at 4°C for future use.

RESULTS AND DISCUSSION

The isolates screened exhibited a range of growth rates from 0.27 to 2.98 mm/day (Fig. 1), with the majority of isolates showing a slower growing habit.

The breakdown of the curve in Fig. 1 into the R– and W–type isolates, as classified by RAPD analysis (Fig.2), shows the existence of a subset of R–type isolates which grow at typical W–type rates (1.6 to 2.2 mm/day). The overlapping of the two growth curves and the existence of fast growing R–types indicate that growth rate alone is an inadequate phenotypic marker for pathotype identification.

All isolates were also classified for colony morphology. Most could be clearly classified as E or F, but around 14% were of INT morphology. If maintained in culture for a further 2–4 weeks many of these INT isolates ultimately acquired an F morphology.

RAPD profiles were generated for all isolates using 3 primers diagnostic for the W– and R–pathotypes. Table 1 shows the comparison of molecular and morphological characters for each pathotype. Approximately 23% of the isolates screened were W–type, and of these almost all were even edged. The R–type isolates were morphologically more diverse, with around 16% scored as INT, and 4% E, indistinguishable from typical W–types. Thus, the only accurate phenotypic marker was F growth, which correlated 100% with an R–type RAPD profile.

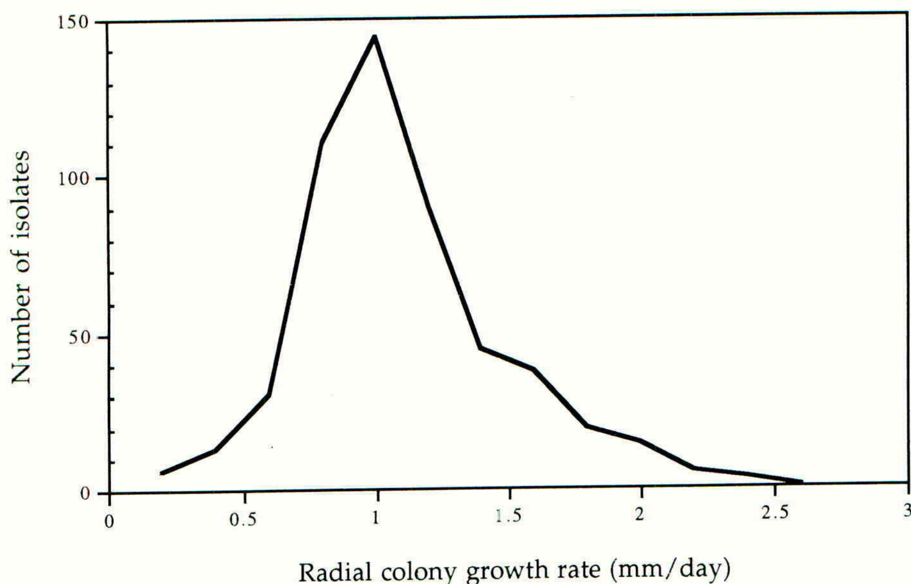

FIGURE 1. Frequency distribution of radial colony growth rates of the 515 field isolates tested. Mean growth rates estimated after 14 days incubation.

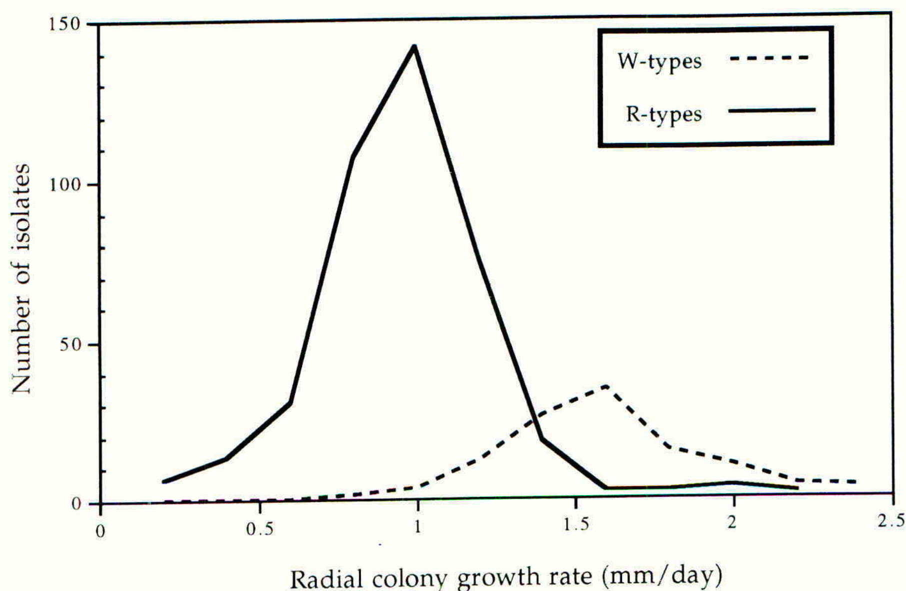

FIGURE 2. Frequency distribution of R– and W–type isolates showing occurrence of R–types with growth rates equivalent to typical W–types.

The F/E R–type isolates, assumed prior to molecular characterisation to be W–types, are most likely strains exhibiting phenotypic instability (Julian *et al.*, 1994). This morphological switch has been observed to occur *in vitro* at a frequency of up to 3% in some isolates (Julian,

1990). The current survey shows that a similar proportion of R–types from the field produce F/E colonies on agar, indistinguishable from W–types. Hence, neither growth rate nor morphology can provide an entirely accurate classification of eyespot populations.

TABLE 1. Comparison of colony morphology characters and molecular methods for pathotype identification.

Molecular Classification	Morphological Characters					
	E		F		INT	
	No of Isolates	%	No of Isolates	%	No of Isolates	%
W–types	111	87	0	0	6	8
R–types	16	13	316	100	66	92
Total	127	100	316	100	72	100

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CROSS-RESISTANCE AMONG DMI-FUNGICIDES AND SENSITIVITY DISTRIBUTIONS OF *SEPTORIA TRITICI* POPULATIONS

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ABSTRACT

Bulk samples of *Septoria tritici* were collected from wheat fields in the UK in 1992, 93 and 94 and tested *in vitro* against cyproconazole and flutriafol. No significant differences in the sensitivity distributions of populations from different years and different regions were observed. The variation in sensitivity between most and least sensitive isolates was a factor of up to 100. Positive cross resistance between cyproconazole and flutriafol was found for entire populations but also for most single isolates, independent of treatment history or regional origin of the samples. Positive cross resistance occurred also between cyproconazole, tebuconazole, flusilazole, propiconazole and triadimenol, when linear correlations were calculated. So far, there is no indication of a decreased sensitivity of *Septoria tritici* against DMI-fungicides.

INTRODUCTION

Septoria tritici (teleomorph *Mycosphaerella graminicola*), is one of the most common foliar pathogens of winter wheat in Northern Europe. It has been controlled successfully for many years by the use of fungicides, primarily by fungicides of the DMI class (inhibitors of C14 demethylation in sterol biosynthesis). As with other pathogens, the development of resistance to DMIs is a potential risk with *Septoria tritici*. As little information is available on the sensitivity distribution to DMIs in *Septoria tritici*, pathogen sensitivity to cyproconazole and flutriafol of more than 500 bulk samples from different regions in the UK was monitored in the years 1992 - 94. In addition, the variability in DMI sensitivity and the cross resistance pattern between cyproconazole, flutriafol and several other triazoles were determined.

MATERIALS AND METHODS

Leaf samples, infected with *S. tritici*, were collected between the end of May and beginning of August in different regions of England and Scotland. The pathogen was isolated from pycnidia and spore suspensions (bulk samples) were used for testing sensitivity to triazoles on fungicide amended Potato Dextrose Agar (PDA), as described by Gisi & Hermann (1994). Dose-response correlations were used to calculate EC 50 values (effective concentrations resulting in 50% inhibition of fungal growth). Two reference isolates were included in the tests: RL2, a very sensitive isolate and S27, an isolate with reduced sensitivity. The original and log-transformed data were tested for normal distribution. The Kolmogorov-Smirnov test and the Mann-Whitney U test were used to compare sensitivity distributions. Cross resistance patterns were analyzed by calculating correlations or linear regressions.

RESULTS AND DISCUSSION

Sensitivity distributions in the years 1992, 1993 and 1994

In 1992, the EC 50 values for cyproconazole ranged from 0.03 to 0.9 mg/l (average EC 50 0.18 mg/l). In 1993, the values ranged between 0.01 and 0.4 mg/l (average 0.15 mg/l) and in 1994, the results were similar to those of 1993 (Figure 1, Table 1). For flutriafol, the EC 50 values varied from 0.08 to 6.3 mg/l in 1992 (average 1.0 mg/l) and from 0.04 to 3.2 mg/l in 1993 (average 0.8 mg/l) (Gisi & Hermann, 1994). The width of the sensitivity distribution (between the lowest and highest EC 50 values) was a factor between 6 and 40 for cyproconazole (Table 1) and between 60 and 80 for flutriafol (data not shown). In all four years, the sensitivity of the samples was clearly higher than that of the reference isolate S27. No significant differences in sensitivity were observed between different years according to the Mann-Whitney U-test. Also, no significant differences in sensitivity were found between samples isolated from different regions of England and between samples isolated from DMI-treated and fields not treated with DMIs (Table 1).

TABLE 1. Sensitivity of *Septoria tritici* to cyproconazole and flutriafol in different years

Year	origin of sample	No. of isolates	EC 50 values (mg AI/l) Mean	Minimum	Maximum	Width of distribution [a]
Sensitivity to cyproconazole						
1991	L. Ashton[b]	18	0.10	0.03	0.18	6
1992	all samples	259	0.18	0.03	0.90	29
1993	all samples	219	0.15	0.01	0.40	40
- ”-	untreated[c]	52	0.18	0.03	0.40	13
- ”-	DMI-treated[c]	162	0.14	0.01	0.40	40
- ”-	East[d]	141	0.14	0.01	0.40	40
- ”-	South[d]	37	0.18	0.02	0.40	20
- ”-	West[d]	41	0.14	0.03	0.35	12
1994		57	0.14	0.05	0.50	10
Sensitivity to flutriafol						
1990	L. Ashton [e]	98	1.2	0.05	11.8	236
1991	L. Ashton [b]	18	0.7	0.10	1.3	13
1992	all samples	252	1.0	0.08	6.3	79

(a): Factor between minimum and maximum value
(b) samples provided by D.W. Hollomon, tested according to SANDOZ methods
(c, d): Samples 1993: (c) from untreated or DMI-treated fields; (d): from different regions in England
(e) Results of D.W. Hollomon (personal communication), EC 50 values transformed from MIC-values, transformation factor derived from reference isolates with known MIC- and EC 50-values

According to the Kolmogorov-Smirnov test, the 1992 and the 1993 data were not log-normal distributed. The 1992 sensitivity distribution may represent a bimodal distribution (Figure 1 A), but this pattern was no longer such obvious in 1993 (Figure 1 B). On the other hand, the 1994 data (Figure 1 C) are log-normal distributed. Since no change in the average as well as minimum and maximum EC 50 values was observed during the three years (Table 1), the bimodal distribution in 1992 is more likely a result of sampling methods rather than an indication of a triazole resistant subpopulation of *Septoria tritici*.

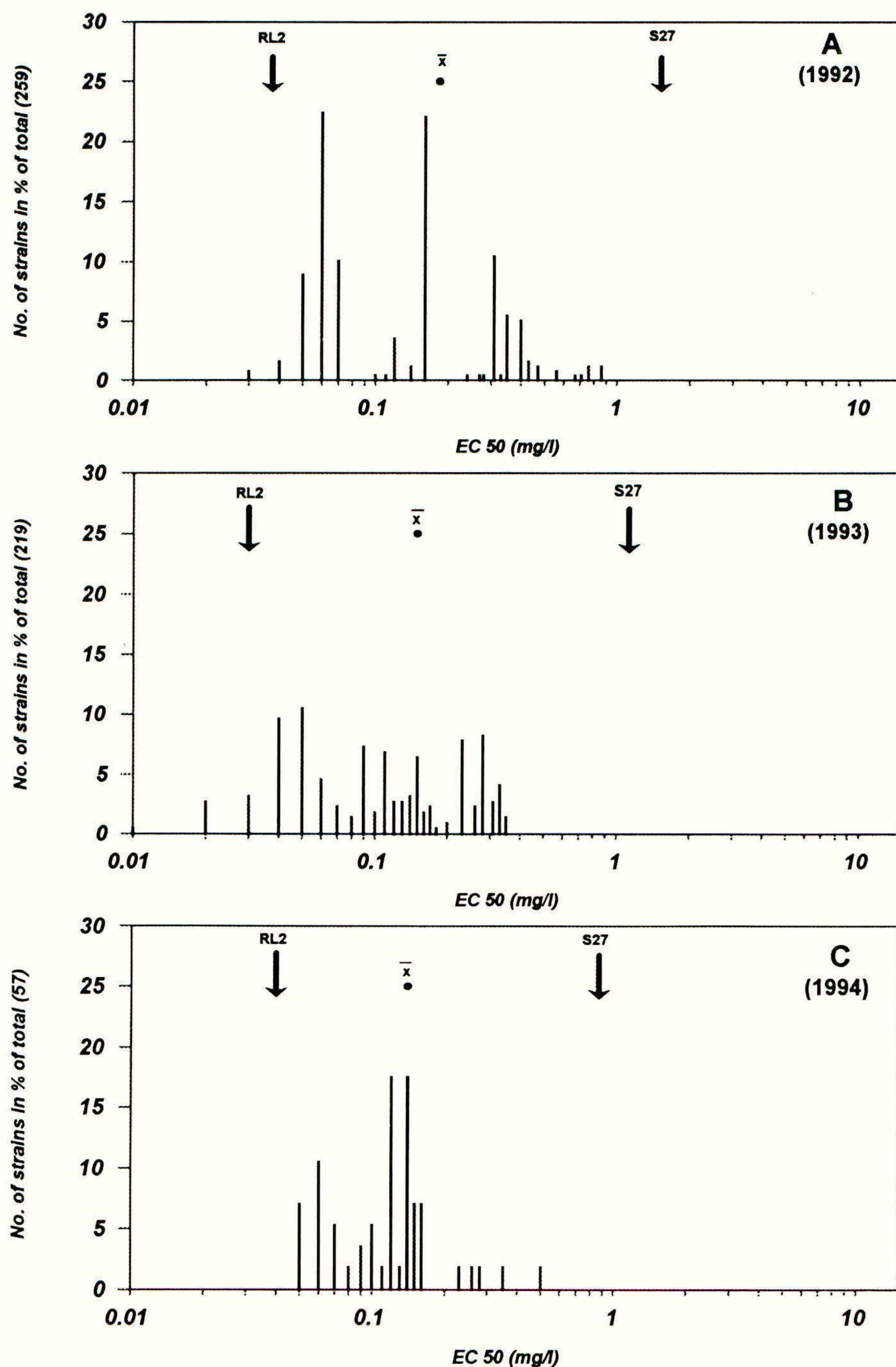

FIGURE 1. Sensitivity distributions of bulk samples of *S. tritici* to cyproconazole isolated from wheat fields in the UK 1992 (A), 1993 (B) and 1994 (C). RL2 and S27 are sensitive and less sensitive reference isolates, respectively, $\overline{x}$ is the mean EC 50 value of the distribution.

If sensitivity distributions to flutriafol between 1990 and 1992 are analyzed (Table 1), no sensitivity shifts can be observed. There is no indication of a decreased sensitivity of *Septoria tritici* against cyproconazole (1991 - 1994) and flutriafol (1990 - 1992). It is an open question whether a shift should have occurred prior to 1990. Bulk samples, as used for sensitivity monitoring, are most likely genetically inhomogeneous but represent a larger part of a field population than single spore isolates. Therefore, bulk samples are expected to show a higher variation of sensitivity compared to single spore isolates. The probability to detect shifts in sensitivity might be lower with bulk samples than with single spore isolates. On the other hand, different sensitivities of single spore isolates should not be overestimated. So far, effective fungicides like cyproconazole provided excellent control of *Septoria tritici* (no correlation between sensitivity and disease severity).

Cross resistance pattern between cyproconazole and other triazoles

Significant positive sensitivity correlations between cyproconazole and flutriafol were found independent of year, origin and treatment history of the samples (Figure 2 F). The sensitivity of 11 single spore isolates was evaluated against different triazoles (Table 2). Cyproconazole, tebuconazole, propiconazole and flusilazole showed similar activity. Flutriafol was about 6 to 10 times less active than cyproconazole (for populations: Gisi & Hermann 1994, for 11 isolates: Table 2) and triadimenol was even less active.

TABLE 2. Sensitivity of *Septoria tritici* isolates from England to triazoles (*in vitro*)

Fungicide	No. of isolates	EC 50 values (mg AI/l)			Width of distribution [a]
		Mean	Minimum	Maximum	
Cyproconazole	11	0.38	0.02	2.1	105
Tebuconazole	11	0.34	0.02	2.1	105
Propiconazole	11	0.23	0.01	1.0	100
Flusilazole	11	0.39	0.02	2.0	100
Flutriafol	11	2.3	0.2	15.0	75
Triadimenol	9	8.4	0.3	22.0	73

(a): Factor between minimum and maximum value

The sensitivity to cyproconazole was plotted versus the sensitivity to other triazoles. The log transformed data followed normal distributions, they were further analyzed by establishing correlations and linear regressions (Figure 2, Table 3). All correlations revealed positive cross resistance patterns between the tested triazole fungicides. The strongest correlations were found between cyproconazole and flutriafol, tebuconazole and flusilazole (Figure 2 A, C, E). Strong correlations were also found for most of the other possible pairwise comparisons (Table 3). Therefore, the tested DMI-fungicides are considered to belong to the same cross resistance group. The present study with *Septoria tritici* does not confirm indications for an inconsistent cross resistance pattern among DMI-fungicides claimed for other pathogens like *Pyrenophora teres* (Nuninger & Staub, 1990).

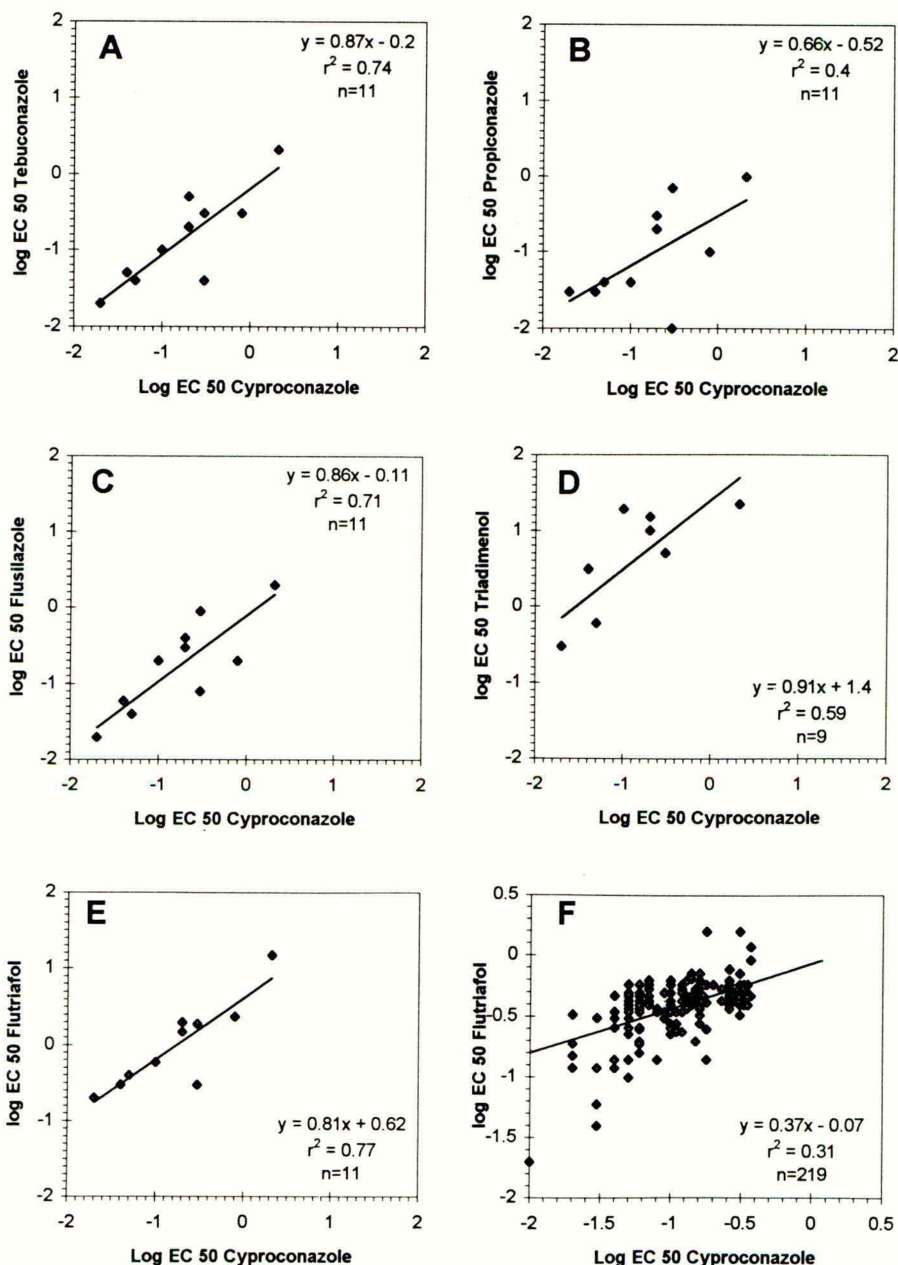

FIGURE 2. Cross resistance patterns between triazole fungicides in *Septoria tritici* Scatterplots of log-transformed EC 50 values of cyproconazole versus: tebuconazole (A); propiconazole (B); flusilazole (C); triadimenol (D); flutriafol (E and F) (E: 11 isolates, F: 219 isolates sampled 1993).

TABLE 3. Cross resistance pattern between triazole fungicides in *Septoria tritici*
Figures are correlation coefficients r (all correlations are significant at $P \leq 0.05$)

	cypro-conazole	tebu-conazole	propi-conazole	flusilazole	flutriafol
tebuconazole	0.86	—			
propiconazole	0.63	0.89	—		
flusilazole	0.84	0.95	0.87	—	
flutriafol	0.88	0.98	0.89	0.92	—
triadimenol	0.77	0.77	0.55	0.88	0.69

When the 11 isolates are used, strong sensitivity correlation between cyproconazole and flutriafol is found (Figure 2 E, $r = 0.88$). On the other hand, a high number of isolates with rather small differences in sensitivity (219 isolates sampled 1993, Figure 2 F) result in a weaker correlation ($r = 0.56$) that is still significant ($P = 0.01$) due to the high degree of freedom. If only a few isolates are used, single outliers result in a weak correlation (e.g. Figure 2 B). Such single isolates should not be used for argumentation against the basic cross resistance pattern that is found when populations are compared. The documented general cross resistance behaviour shows, that a possible sensitivity shift in *Septoria tritici* would affect the efficacy of all tested triazoles in the same way.

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MULTIPLE FUNGICIDE RESISTANCE IN *BOTRYTIS CINEREA* FROM COMMERCIAL GREENHOUSES IN SOUTHEASTERN SPAIN

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ABSTRACT

Forty nine greenhouses of vegetable crops were surveyed in southeast Spain in December 1992 to detect fungicide resistance to benzimidazoles, dicarboximides, and the mixture of carbendazim and diethofencarb in *B. cinerea*. Out of 260 isolates collected, 28% were sensitive to benomyl and procymidone (Ben^SPrc^S), 16% were benomyl-resistant and procymidone-sensitive (Ben^RPrc^S), 8% were Ben^SPrc^R and 49% were Ben^RPrc^R. Three triple resistant isolates ($Ben^RPrc^RCD^R$) were found in two neighbouring greenhouses that were sprayed with a mixture of carbendazim and diethofencarb. These three isolates and twenty nine selected by their phenotype of benomyl and procymidone sensitivity were characterized *in vitro*. Sensitive isolates grew slower on PDA than resitant isolates, but their sporulation, spore germination and length of germ tube were similar for all groups. Benzimidazole- and dicarboximide-resistant isolates produced the greatest number of sclerotia while benzimidazole-resistant produced the largest sclerotia.

INTRODUCTION

Grey mould disease, caused by *Botrytis cinerea* Pers., causes severe losses in winter crops greenhouses in southeastern Spain (Belda *et al.*, 1994). Winter crops are tomato, squash, bean, pepper, cucumber and eggplant, all of them susceptible to *B. cinerea* (Smith *et al.*, 1988). This potential damage compels growers to protect their crops in the period they feel the environment is more favourable for the disease development, usually from November to March. Fungicides used include benzimidazoles, dicarboximides and dichlofluanid, which are applied on average every seven days. In the last four years, the mixture of carbendazim and diethofencarb has been commercialized and its use is becoming popular.

Resistance to benzimidazoles in *B. cinerea* from greenhouses has been widely reported (Porta-Puglia, 1978), as well as resistance to dicarboximides (Panayotakou & Malathrakis, 1983; Wang *et al.*, 1986), and double resistance to both groups of fungicides (Rewal *et al.*, 1991; Moorman & Lease, 1992). Recently, some isolates have been found which show multiple resistance to benzimidazoles, dicarboximides and the mixture of carbendazim and diethofencarb (Elad *et al.*, 1992). However, little is known about the incidence of fungicide resistance to any of these groups in this region of southeastern Spain. The research described here was carried out to determine the presence of resistance to benzimidazoles, dicarboximides and the mixture of carbendazim and diethofencarb in *B. cinerea* in protected crops, and to evaluate some *in vitro* characteristics related to the fitness of the resistance.

MATERIALS AND METHODS

Survey of fungicide resistance of isolates of *B. cinerea*

A total of 260 isolates from 49 commercial greenhouses of usual winter crops were collected in December 1992. Between three and seven pieces of either stems, leaves, flowers or fruits from different plants showings symptoms of grey mould disease were sampled in each greenhouse. To isolate *B. cinerea*, small pieces taken from the edges of lesions were placed on potato dextrose agar (PDA) medium amended with 0.5 g/l streptomycin sulphate in Petri dishes, and incubated on a laboratory bench at 20-25°C. Growing mycelium was transferred to PDA slants and stored at 4°C.

Tests for fungicide resistance were done by transfer of a 6-mm-diameter plug of actively growing mycelium to each of the three sectors of a plate containing Oxoid PDA medium supplemented with fungicide. Fungicides were technical grade of benomyl (Aragonesas, SA), procymidone (Agrocros, SA), and the mixture (1:5) of carbendazim (Kemichrom) and diethofencarb (Sumitomo). They were dissolved in acetone and then added to the medium to have a final concentration of 5 mg/l of the first two fungicides or 6 mg/l of the mixture, and 5 ml/l of acetone. Control plates included 5 ml/l of acetone. Plates were incubated at 20°C in the dark. After three days, isolates were considered as resistant if they grew on fungicide-amended media, and as sensitive if they failed to do so. There were two replicates of each test.

Fungicide sensitivity

Fungicide concentration that reduced fungal growth by half (LC_{50}) was determined for each isolate in presence of benomyl, iprodione (Rhône-Poulenc) and the mixture of carbendazim+diethofencarb (1:5). An automated quantitative method (AQ) was applied as described previously (Raposo *et al.*, 1993) to establish the dosage-response curves. In this method, each isolate was grown in potato dextrose broth (PDB; Difco) in the wells of a microplate with different fungicide concentrations. After 46 h of incubation at 20°C in darkness, the total fungal growth was determined by measuring absorbance at 492 nm wavelength. The wells of the microplate were filled to have a spore concentration of 10 spores/μl in a final volume of 100 μl. Spores had been obtained earlier on PDA cultures grown as usual in a growth chamber for 7 days (16 h photoperiod; 25°C under fluorescent light and 21°C in the dark). Spores were stored at -20°C in a 20 % glycerol solution until use. Benomyl fungicide used in this test was commercially formulated (Zetamilo 50WP, ICI-Zeltia), and the microplates containing benomyl beyond its solubility were incubated under continuous agitation. All LC_{50} values were determined at least twice.

Characterization *in vitro*

A total number of 31 isolates were selected to determine some *in vitro* properties and compare them on the basis of their phenotype for fungicide resistance.

Linear growth rate of each isolate was determined by transfer of an actively-growing mycelium plug of 6 mm diameter to PDA plates. After two days of incubation at 20°C in the darkness, two perpendicular diameters of the colony were measured. They were averaged after subtraction of the 6 mm of the

plug size. The growth rate was expressed in mm/h units. Each isolate was cultured in four plates and the experiment was repeated.

To determine sclerotia production, plates handled as described above were incubated for three weeks. The number of sclerotia (number/cm^2 of colony) and the mean area of sclerotia in the colony (mm^2/cm^2) on each of the four plates were determined by image analysis (CUE-2 version 4.5 software; Galai Production Ltd.). The experiment was repeated.

Sporulation, percentage of germinated spores and their length of germ tube were determined for a set of seven isolates. They represent a sample of each phenotype for benzimidazole and dicarboximidde resistance and they were sensitive to the mixture of carbendazim and diethofencarb. To quantify the sporulation, each isolate was grown on four plates for 10 days at 20°C in dark. The colony, that always covered the whole plate, was cut in small pieces, placed in a volume of 150 ml of a solution of 1 % Tween-80 and sonicated for 5 min. The number of conidia was counted with a haematocytometer in four subsamples per plate, and the number/cm^2 was calculated. To determine percentage of germination and germ tube length, spores were obtained from PDA cultures as described for the determination of fungicide sensitivity. A filtered spore suspension was obtained and added to tubes (15x65 mm) with 3 ml of double concentrated sterile PDB (48 g/l; Difco) to have a total concentration of 20,000 spores/ml. After they were germinated for seven h at 20°C in the darkness, four 30 μl drops were mounted on slides and the number of germinated spores out of 50 and the length of germ tubes of 25 conidia were measured in each drop. Estimated values were the mean of these four counts. The experiment was repeated.

All data were subjected to statistical analysis performed with SAS ver. 6.03 for micro-computers, procedure GLM. Means were compared by the protected LSD method (P= 0.05).

RESULTS AND DISCUSSION

Survey of fungicide resistance

Dicarboximide- or benzimidazole- resistant isolates of *B. cinerea* were found in each of the 49 surveyed greenhouses. In two neighbouring greenhouses, that were treated with the mixture of carbendazim and diethofencarb in the last few years, three isolates were found which were resistant to the mixture as well as to the other two groups of fungicides. The formulated mixture has been commercialized in Spain only since 1990, so the detection of the triple resistant isolates indicates a rapid development of this resistance. Resistance to benzimidazoles and the mixture of carbendazim and diethofencarb had been found in 1988 in Israel (Katan *et al.*, 1989), two years after the fungicide was tested in protected cucumbers. Triple resistant isolates were found in commercial greenhouses in the following year (Elad *et al.*, 1992).

Out of 260 isolates collected in southeastern Spain, 64% were resistant to benomyl and 56.5% were resistant to procymidone. The double resistant phenotype was the most frequent (48%) (Ben^RPrc^R), followed by the double sensitive (Ben^SPrc^S) (28%). Frequency of Ben^RPrc^S phenotype was 16% and of Ben^SPrc^R was 8%. Resistance to procymidone in isolates of *B. cinerea* collected in a broader area in Spain in 1982 was 67% (Fraile *et al.*, 1986), but this is the first survey in which multiple resistance for the commonest applied fungicides was detected.

Fungicide sensitivity

Dicarboximide-resistant isolates had LC_{50} values for fungal growth in the range of 1.5-3.9 mg/l iprodione, and the sensitive had LC_{50} values lower than 0.5 mg/l. Benzimidazole-resistant had LC_{50} values in the range of 105-324 mg/l, whereas benzimidazole-sensitive were in the LC_{50} range of 0.04-0.07 mg/l. One isolate had LC50 of 13.07 mg/l. Correspondence between LC_{50} values obtained by the AQ assay and the conventional method (RG) of measuring radial growth of mycelium on fungicide-amended medium has been established for iprodione and the mixture of carbendazim and diethofencarb (Raposo *et al.*, unpublished). For iprodione this was $\log Y = -0.392 + 1.04 \log X$, where X and Y are the LC_{50} value determined, respectively, by the AQ method and the RG method. Thus, dicarboximide-resistant isolates are considered as low resistant carrying the Daf1LR allele (Faretra & Pollastro, 1993). No isolates high resistant to dicarboximides have been found. Assuming a similar trend between LC_{50} values for benomyl, benzimidazole-resistant would be considered as high resistant carrying the Mbc1HR allele and sensitive to N-phenylcarbamate fungicides (Faretra & Pollastro, 1993). However, one isolate would be low resistant carrying the Mbc1LR allele (Faretra & Pollastro, 1993). All sensitive isolates for the mixture of carbendazim and diethofencarb had LC_{50} values in the range of 0.01-0.15 mg/l carbendazim and 0.01-0.15 mg/ml diethofencarb, and the resistant 15-26 mg/ml carbendazim and 75-130 mg/ml diethofencarb.

Characterization *in vitro*

Isolates exhibited a high variability for growth rate at 20°C, sclerotial production and sporulation measured on PDA media. Fungicide sensitive isolates had a mean linear growth rate significantly lower than those of the resistant isolates (Table 1), while dicarboximide-resistant isolates had the highest values. Benzimidazole-resistant phenotypes had a mean linear growth rate between the extreme values (difference between them of 0.162 mm/h). These observations are unusual, since in other studies mean growth rates were similar for benzimidazole-sensitive and -resistant (Beever *et al.*, 1989), dicarboximide-sensitive and -resistant (Pommer & Lorenz, 1982), and for double resistant and sensitive (Hsiang & Chastagner, 1991). Katan (1982) and Fraile *et al.* (1986) found differences for resistance to dicarboximides, but it was the sensitive isolates which grew faster.

Mean number of sclerotia produced per cm^2 of colony on PDA was significantly greater (up to 71%) for the double-resistance phenotype, whereas it was similar among any of the other fungicide-resistant groups. Mean sclerotial size was larger for the benzimidazole-resistant group, but did not differ between the other groups. No measurement was done for any isolate in the triple resistant group. Sclerotia are considered the most important structure for surviving in *Botrytis* species (Coley-Smith, 1980). Implications that number and size of sclerotia have on their survival or viability are unknown, and require further research to establish a correlation between these characteristics.

Each of the seven isolates tested to quantify their sporulation sporulated well on PDA media at 20°C in dark, and there were no significant differences in sporulation between isolates. Similar results have been reported in other studies when comparing differences within a specific fungicide resistant group (Fraile *et al.* 1986; Katan, 1982; Moorman & Lease, 1992), but not for the double resistance to benzimidazole and dicarboximide (Hsiang & Chastagner, 1991), which showed only half the sporulation of the sensitive strain. Percentage of germinated conidia and length of their germ

tube were similar among these seven tested isolates.

In vitro characteristics measured here are related to the fitness of the isolates. According to their sporulation capacity, fitness of the benzimidazole- and/or dicarboximide isolates is similar. However, linear growth rate values suggest that the triple sensitivity group is less fit than the other groups. This could be explained on the basis of the long duration of the selection pressure for both groups of fungicides, since applications have not been interrupted in these years. Finally, the high frequency of benzimidazole-resistant found in this survey indicates that applications with this active ingredient should be avoided, and that a proper grey mould management program
to prevent an increase in the frequency of the triple resistant phenotype is needed.

TABLE 1. Linear growth rate, sclerotia production, sporulation and viability of spores for fungicide-resistance phenotypes in isolates of *Botrytis cinerea*.

Phenotype	Growth rate (mm/h)	Sclerotia		Number spores/ cm²	Germination (%)	Germ tube length (μm)
		no./cm²	area/cm²			
$Ben^SPrc^SCD^S$	0.987 a	0.53 a	6.225 a	458345 a	71.0 a	20.41 a
$Ben^RPrc^SCD^S$	1.122 b	0.56 a	8.129 b	938439 a	64.3 a	26.29 a
$Ben^SPrc^RCD^S$	1.149 c	0.61 a	6.231 a	502991 a	87.6 a	29.59 a
$Ben^RPrc^RCD^S$	1.105 b	0.97 b	6.341 a	323325 a	90.1 a	28.87 a
$Ben^RPrc^RCD^R$	1.127bc	-	-	-	-	-

Means followed by the same letter within a column are not significantly different by the protected LSD test (P=0.05).

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