POSTER SESSION 8D

RESISTANCE OF PESTS AND PATHOGENS TO PESTICIDES

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Insecticide resistance in Egyptian strains of *Bemisia tabaci*

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ABSTRACT

A strain of *Bemisia tabaci* ('B'-biotype) collected from cotton and vegetable crops at the beginning of the growing season in Egypt displayed marked resistance to a carbamate (carbosulfan) and moderate resistance to a pyrethroid (cypermethrin). It displayed no resistance to an organophosphate (profenofos) or to the neonicotinoid imidacloprid. Another strain, collected at the end of the growing season from the same location, differed markedly in its response. In this strain, resistance to carbosulfan remained high but resistance to profenofos and cypermethrin increased. Slight resistance to imidacloprid was detected. Neither strain showed resistance to the insect growth regulator pyriproxyfen. The resistance profiles of these strains were compared to that of a typical Israeli field strain ('Q' biotype) and are discussed with reference to their collection date and the patterns of insecticide use in those countries. The ability of Egyptian 'B' biotypes to introgress with Israeli 'Q'-biotypes was examined, and the resistance characteristics of the resulting offspring were investigated. The consequences of introgression between co-existing biotypes with different resistance profiles are considered.

INTRODUCTION

For the past twenty-five years, *Bemisia tabaci* (Hemiptera: Aleyrodidae) control in Egypt has been based almost exclusively on organophosphates, carbamates and pyrethroids. Little work quantifying resistance in *B. tabaci* in Egypt has been published, but it is clear that it is now a major constraint to both cotton and vegetable production (Abdallah, 1991). Ten to twelve insecticide applications per season are common in the cotton crop and in tomatoes, the risk of whitefly-mediated virus transmission has led farmers to apply organophosphate / pyrethroid insecticides two or three times per day. Despite this, the transmission of tomato yellow leaf curl virus (TYLCV) often results in complete loss of the autumn tomato planting.

In many parts of the world, the epidemiology of resistance in whiteflies can be considered within the context of a single, ubiquitous whitefly biotype; the 'B' biotype. This is a highly competitive variant of *Bemisia tabaci* and often supplants indigenous forms. In countries surrounding the Mediterranean, however, the indigenous 'Q' biotype is also present. These races are morphologically identical but differ in biochemical, physiological and life-history traits (Muniz and Nombela, 2001; Horowitz *et al.*, 2001). To date, hybrid forms appear rare, but their possible introgression is clearly of great importance for the spread of resistant traits between biotypes.

This paper provides an analysis of the resistance displayed by Egyptian 'B'-biotype *B. tabaci* strains to traditional, broad spectrum compounds in current use and to also to imidacloprid (recently registered in Egypt) and pyriproxyfen (under trial). Temporal changes in resistance patterns between strains are noted and discussed. We compare these data to the resistance profiles exhibited by a 'Q'-biotype *B. tabaci* from the neighbouring state of Israel. We then examine the potential of these 'B' and 'Q' biotypes to introgress and thereby illustrate the risk of resistance characteristics transferring between such sub-populations.

METHODS AND MATERIALS

Insects

An insecticide susceptible strain (SUDS), collected from cotton fields in Sudan in 1978, was used as a reference for evaluating the magnitude of resistance in field-collected strains. At the beginning of the cotton and vegetable growing seasons (1/5/00), whiteflies were collected from a cotton field in Qaliobia, Egypt. At the end of the cotton season (1/12/2001) this site was revisited, and a further collection made. The Israeli strain was provided by the Volcani Center, Israel. The biotype of Israeli and Egyptian populations was ascertained by comparing their esterase banding patterns (Gorman and Denholm, 2000).

Introgression between biotypes

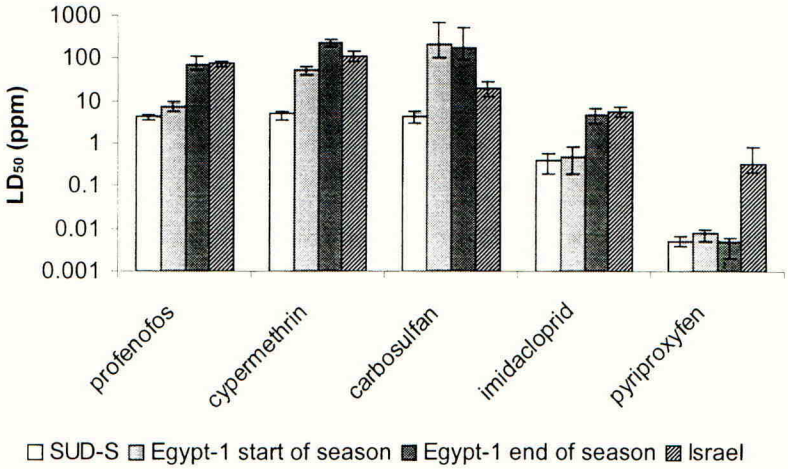
Single virgin females and single males were placed together on excised leaves in leaf boxes. The number of offspring produced was noted 25 days later. Those that had reached the adult stage were sexed. The haplodiploid breeding system of *B. tabaci* means that successful mating is indicated by the presence of female offspring. These offspring were assayed against profenofos in order to ascertain the resistance characteristics of these hybrids.

RESULTS AND DISCUSSION

The early season collection of Egyptian whitefly displayed little or no resistance to the organophosphate profenofos. In comparison, the Israeli strain and the Egyptian strain collected at the end of the season, were ca 20-fold resistant. The early season Egyptian strain exhibited ca. 10-fold resistance to cypermethrin whilst the Israeli strain exhibited 20-fold resistance. In the Egyptian strain collected at the end of the season, resistance to cypermethrin had increased to ca. 50-fold. Egyptian strains were 20 to 50-fold resistant to carbosulfan, regardless of time of collection whilst Israeli strains displayed no resistance. Although the early season collection from Egypt was susceptible to imidacloprid, the late season collection showed the same slight resistance as the Israeli strain (ca 6-fold). Pyriproxyfen proved very effective against the nymphs of all the Egyptian strains, whilst the Israeli strains displayed strong resistance (68-fold) (Figure 1).

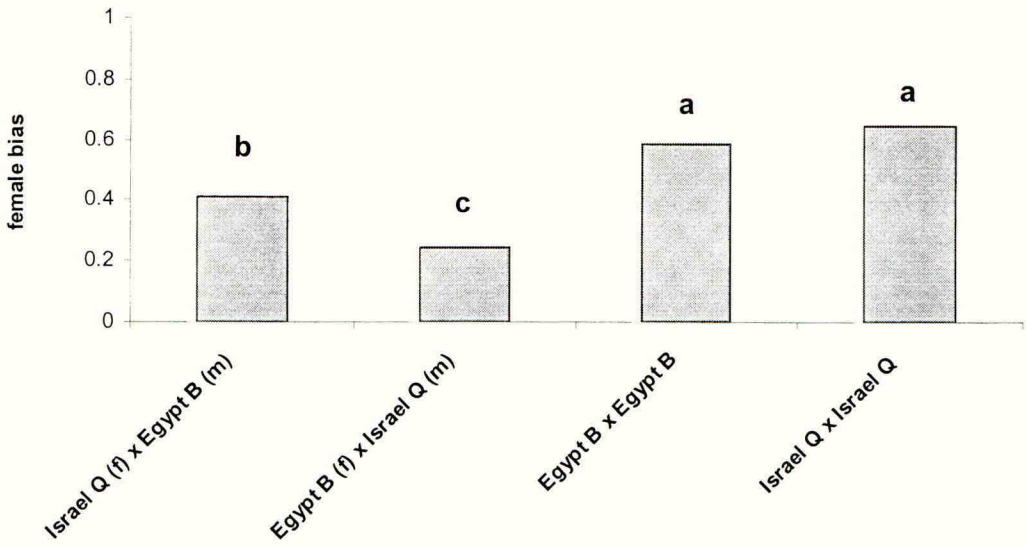
Single pair mating between biotypes was successful but, using sex ratio as an indicator of mating success, not as successful as mating within biotypes (Figure 2). In the case of their response to profenofos, the offspring exhibited resistance characteristics intermediate to that of their parents (Figure 3).

Figure 1. Relative LD_{50} values (with 95% confidence limits) of Egyptian and Israeli strains



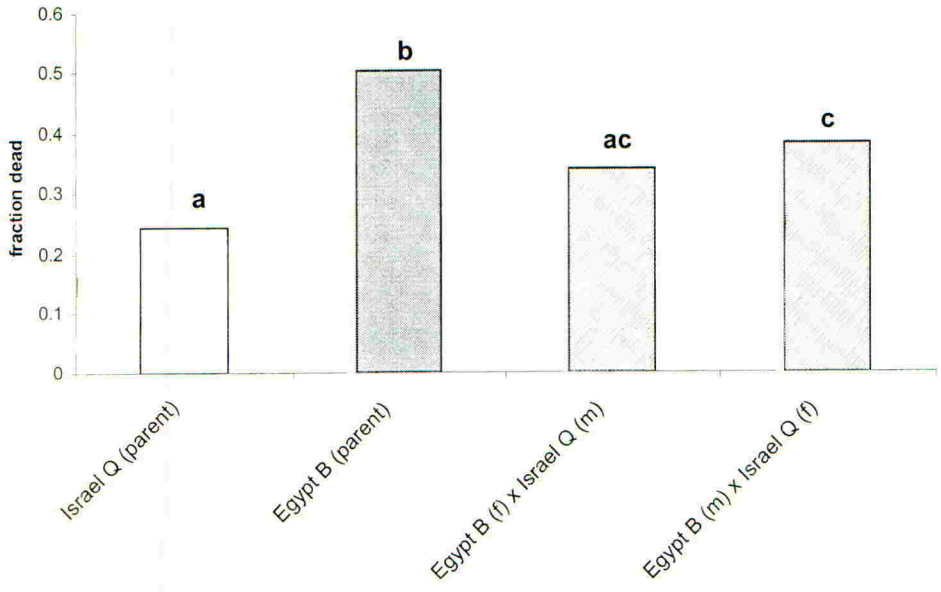
Non-overlapping confidence limits denote significant differences between means ($p < 0.05$)

Figure 2. Female bias of reciprocal crosses between 'B' (Egyptian) and 'Q' (Israeli) biotypes



Bars annotated with different letters denote significant differences by $\chi 2$ ($F>3.84$, $p<0.05$)

Figure 3. Response of resistant (Israel 'Q' biotype□) and susceptible (Egypt 'B' biotype ■) parents and their offspring (▨) to a discriminating dose of 8 ppm profenofos.



Bars annotated with different letters denote significant differences by $\chi 2$ ($F>3.84$, $p<0.05$)

DISCUSSION

Egypt appears to be experiencing the same kind of resistance-mediated *B. tabaci* problem that beset Israel two decades ago. Until the 1990s, most vegetable and flower growers in Israel seldom used even simple techniques to combat insecticide resistance (such as alternation of two or more insecticides). As in Egypt, tomato growers found that, in order to combat *B. tabaci* transmitted geminiviruses, one to two insecticide applications per day had to be applied and that even then, many plots were totally infected with TYLCV (Horowitz *et al*, 1998). At that time, Israel implemented a highly coordinated resistance management strategy that utilised some of the new chemistries (buprofezin, pyriproxyfen, neonicotinoids etc) in defined alternations. As a result of the fact that they are not yet compromised by resistance, and are less detrimental to beneficial insects, only one or two sprays per season are now considered necessary to control whiteflies on cotton (Denholm *et al*, 1998).

The results of the current study suggest that a similar strategy might now be possible in Egypt. Despite the intensive use of organophosphates against Egyptian whitefly for over two decades, the early season collection was susceptible to profenofos. Cypermethrin was more highly resisted, but even in this instance, the differential response between the late and early season collection may suggest that the efficacy of this compound is partially protected by some combination of ecological or biological factors that conspire over the winter period to offset the resistance selected for during the growing season. Only the carbamates appear to be truly

compromised. Our results show that pyriproxyfen is likely to be extremely effective, and that imidacloprid is not yet strongly resisted. The recent or imminent registration of these new chemistries, in tandem with the remaining efficacy of older products, represents an exciting opportunity to carefully manage resistance within the frameworks of insecticide rotation and integrated pest management.

We suggest that the institution of simple alternation strategies involving the use of organophosphates and pyrethroids in combination with more novel compounds should help tackle the whitefly problem whilst conserving insecticide susceptibility.

In a comparison of inter and intra-biotype crosses, the latter were more likely to be successful (i.e to produce at least some female offspring). Moreover, of the inter and intra-biotype crosses that were successful, the latter also produced the greatest proportion of females. We suggest therefore, that inter-biotype crosses are not as successful as intra-biotype crosses, but that they are possible. We have also illustrated that, when they do occur, resistant traits can be successfully transferred between populations. In the case of profenofos resistance, this resistance is partially dominant and so is likely to be selected for rapidly in a new hybrid population.

We further suggest therefore, that as it is clear that 'B' and 'Q' biotypes can introgress, there is a risk that the development of resistance in Egypt could be accelerated by the immigration of other biotypes exhibiting increased resistance to traditional and/or novel compounds. In order to maximise our ability to manage resistance, it is imperative that Egypt continues to monitor resistance in its indigenous whiteflies and to screen for the immigration of additional biotypes with different resistance profiles.

ACKNOWLEDGEMENTS

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Chlorfenapyr resistance in *Helicoverpa armigera* in Australia

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ABSTRACT

Chlorfenapyr has been registered for use against *Helicoverpa armigera* on cotton in Australia since 1997 and is a valuable component of the Australian *Helicoverpa* resistance management strategy. Baseline susceptibility data was accumulated some years prior to registration. During the 1997/98, cotton season, it was evident however, that a low frequency of cotton *H. armigera* (3 %), were resistant to chlorfenapyr and this figure has progressively increased to approximately 17% in 2002. The speed with which *H. armigera* larvae developed resistance was as a result of cross-resistance to high level pyrethroid resistance. Resistance mechanism studies indicate a common esterase mediated resistance mechanism between chlorfenapyr and pyrethroid resistance.

INTRODUCTION

The cotton bolloworm *Helicoverpa armigera* (Hübner) is a serious pest of cotton and other summer crops in Australia, where it has a long history of insecticide resistance to older chemistry (DDT, pyrethroids, carbamates, organophosphates and endosulfan). Chemical insecticides are currently essential for the control of *H. armigera* and given problems with transgenic cotton in Australia, are likely to remain so for the foreseeable future. Insecticide resistance in *H. armigera*, however, is a major threat to the economic production of cotton in Australia.

The development of resistance has been delayed by an *Helicoverpa* resistance management strategy and the registration of new insecticides with novel modes of action. The registration of the pyrrole compound chlorfenapyr for *H. armigera* control has therefore been very important to the Australian cotton industry because it involves a compound with a new mode of action. Chlorfenapyr interferes with insect respiration, functioning as an uncoupler of oxidative phosphorylation. Since these compounds are activated and metabolised via microsomal oxidation, it was considered unlikely that H. armigera, which seems to rely on esterase mediated detoxification (Gunning *et al.* 1996), would develop resistance.

The Australian *Helicoverpa* insecticide resistance management strategy is monitored by an insecticide resistance monitoring programme and on registration in 1997, chlorfenapyr was incorporated into the resistance screen. Baseline toxicity studies against *H. armigera* were completed during 1991-1992 when chlorfenapyr was still an experimental compound. The purpose of the current work is to report the development of resistance in Australian *H. armigera* to chlorfenapyr, cross-resistance and an esterase mediated resistance mechanism.

METHODS AND MATERIALS

Insects

A laboratory susceptible strain of *H.armigera*, as well as field susceptible strains, were used for baseline toxicity studies. Chlorfenapyr-resistant strains were obtained by selecting fenvalerate resistant populations of *H. armigera* (40 and 100 fold) with chlorfenapyr.

Bioassay

Standard third instar contact toxicity bioassys were used. Technical grade chlorfenapyr, (4-bromo-2-(-4chlorophenyl)-1-(ethoxymethyl)-5-(trifluromethyl) pyrrole-3-carbonitrile was dissolved in acetone and serially diluted concentrations prepared. Larvae were treated with 1µl of solution. After dosage, the test larvae were held at 25° C with adequate food. Mortality was assessed 72h after treatment. The data were analysed by probit analysis. Resistance factors were calculated as the ratio of the resistant LD_{50} / susceptible LD_{50}.

Esterase Activity

Total esterase activity of susceptible and resistant larval homogenates were detected using 1-naphthyl acetate as a substrate and using kinetic assays (Gunning *et al*, 1996). Larvae were homogenised in 2M phosphate (pH 7.0) with 0.01% Triton X-100 (50 µl per 4mg/insect tissue) and chlorfenapyr added (in 2µl acetone). The chlorfenapyr homogenates and controls were incubated at 25° C for 60 mins. Aliquots (10 µl) were transferred to a microplate and 240µl of phosphate buffer (pH 6) containing 0.6% fast blue salt RR and 1.86% 1-naphthyl acetate were immediately run on a BioRad 3550 microplate reader in kinetic mode at 450mn.

Electrophoresis

Esterase activity was also examined by electrophoresis (Gunning et al, 1996). Aliquots (10µl), of larval homogenates incubated with chlorfenapyr were loaded into wells of polyacrylamide gels. Gels were run at 250 V and maximum current (at 5° C). Gels were stained for esterase activity.

RESULTS

Baseline data

Baseline data studies with chlorfenapyr, (Table 1) showed susceptible strains, had an excellent dose response relationship, indicating that topical application of chlorfenapyr was a suitable bioassay method for *H. armigera* larvae. Slope values ranged from 1.7 – 3.9. Whilst the reference laboratory strain was most susceptible to chlorfenapyr, LD_{50}'s of field strains ranged from 0.17 – 0.47 µg/larva. For routine resistance monitoring, the discriminating dose was fixed at 5µg/larva (applied by topical application), which exceeded the highest LD_{99} of field reference populations in 1991 – 1992.

Table 1. Baseline response of third instar *Helicoverpa armigera* to chlorfenapyr

Population	LD_{50}(fiducial limits) µg/larvae	Slope	χ^2	$LD_{99.9}$
lab. Susceptible	0.18 (0.15-0.21)	3.1	4.9	1.75
Emerald, 1991	0.47 (0.41-0.55)	3.9	2.4	2.83
Namoi, 1991	0.55 (0.46-0.54)	3.4	2.6	4.97
Warren, 1991	0.15 (0.11-0.23)	1.8	4.8	4.60
Gwydir, 1991	0.17 (0.14-0.21)	3.2	3.2	1.78
Tamworth, 1992	0.38 (0.32- 0.44)	1.7	3.2	4.30
Namoi, 1992	0.19 (0.16-0.24)	3.1	3.3	1.85
Emerald, 1992	0.20 90.17-0.26)	2.9	2.2	1.82
Tamworth, 1992	0.21 (0.18 – 0.24)	3.1	3.2	1.94
Gwydir, 1992	0.25 (0.23 –0.29)	3.0	2.8	2.22
Warren, 1992	0.23 (0.19-0.28)	2.6	2.5	3.91

Resistance monitoring

Chlorfenapyr was incorporated into the *H. armigera* resistance monitoring screen on first commercial use of chlorfenapyr during 1997/98. Resistance frequency was calculated from discriminating dose survival. Chlorfenapyr resistance frequency in Queensland and New South Wales cotton *H. armigera* populations for 1997-2002 are shown (Figure 1). Within one year of limited commercial use, *H. armigera* larvae resistant to chlorfenapyr were detected. Chlorfenapyr resistance frequency in *H. armigera* has progressively increased from approximately 3% in 1997/98 to 17% in 2001/02.

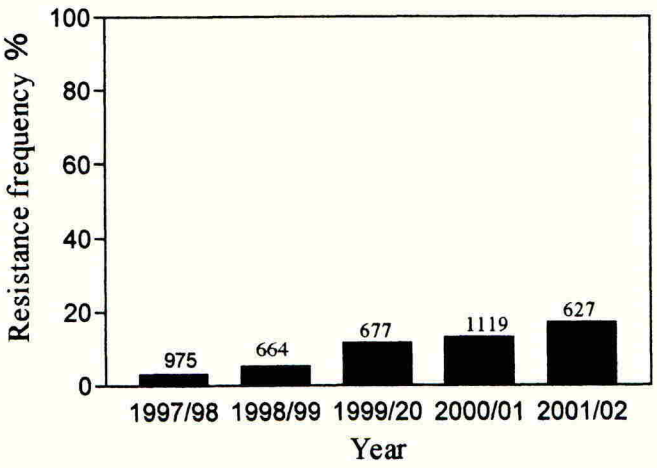

Figure 1. Chlorfenapyr resistance frequency in Australian *Helicoverpa armigera*. Larvae (Numbers on the bars are actual numbers of larvae tested)

Cross Resistance

The very rapid build-up of resistance to chlorfenapyr in *H. armigera* suggested cross-resistance to another insecticide and we suspected pyrethroid resistance as the cause. Chlorfenapyr resistance increase coincided with a period of increasing pyrethroid resistance. During 1997 – 2001, the fenvalerate resistance factor in cotton populations of *H. armigera*, increased from approximately 20 fold to 40 fold. To test this hypothesis, we bioassayed fenvalerate selected *H. armigera* populations (10, 46 and 150-fold with chlorfenapyr (Table 2).

A population showing a low level of fenvalerate resistance (10-fold) was indistinguishable in response to chlorfenapyr from susceptible populations. A 46-fold fenvalerate resistance population, however, showed selection of resistance to chlorfenapyr and a 7-fold resistance factor. The population nonetheless, had considerable heterogeneity of response toward chlorfenapyr (slope 0.9). A population 100 fold resistant to fenvalerate had greatly increased resistance to chlorfenapyr (56 fold) and homogeneity of response (slope 2.7).

Table 2. Response of fenvalerate selected *Helicoverpa armigera* larvae to chlorfenapyr

Population µg/larvae	LD_{50} (fiducial limits)	Slope	χ^2	RF*
lab. Susceptible	0.18 (0.15-0.21)	3.1	4.9	0
fen v. 10 fold	0.47 (0.41-0.55)	3.9	2.4	0
fenv. 46 fold	1.3 (0.87-0.21)	0.9	10.5	7.2
fenv. 100 fold	10.1 (8.3-12.3)	2.7	1.3	56

*RF - resistance factor calculated from the ratio of LD_{50} resistant/ LD_{50}susceptible

Esterase inhibition

Since pyrethroid resistance in field populations of *H. armigera* is largely due to the overproduction of specific esterase isoenzymes which sequester and hydrolyse pyrethroids (Gunning *et al*, 1996), the possibility of esterase mediated resistance to chlorfenapyr was investigated, using a number of chlorfenapyr susceptible and resistant field strains of *H. armigera* (Figure 2). While esterase from susceptible populations was clearly unaffected by incubation with any concentration of chlorfenapyr, resistant populations showed esterase inhibition with increasing concentration of chlorfenapyr. Esterase inhibition occurred within a concentration range of 0.001 – 0.1 mM chlorfenapyr. At the highest concentration, up to 80% of esterase activity was inhibited in resistant strains. Inhibition was greatest in the most highly resistant strain.

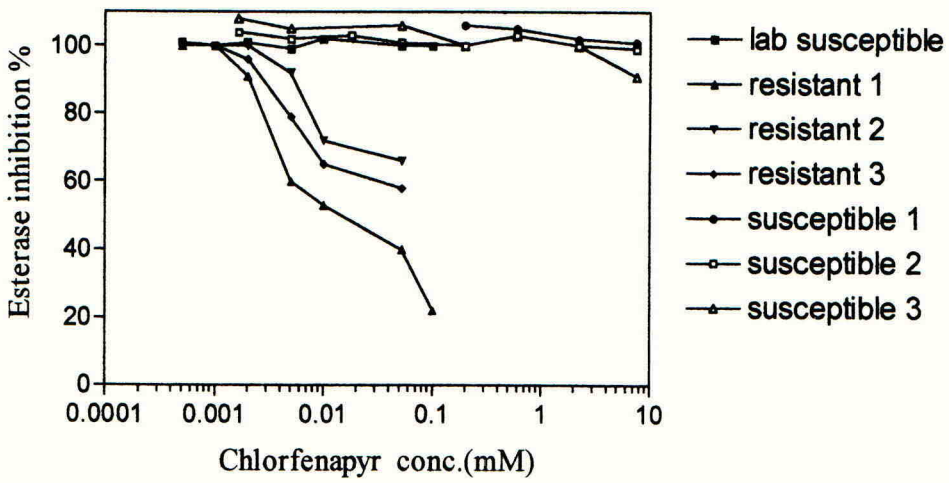

Figure 2. Inhibition of H. armigera esterase by chlorfenapyr in a laboratory susceptible, three field susceptible and three resistant strains.

Electrophoresis

Polyacrylamide electrophoresis showed that *H. armigera* esterases (that bind to pyrethroids, see arrow), were inhibited by increasing concentrations of chlorfenapyr (Figure 3).

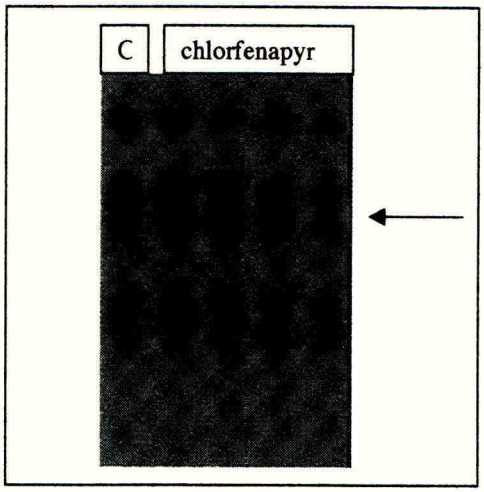

Figure 3. Polyacrylamide gel showing the effects of increasing concentrations of chlorfenapyr (0.025 – 0.5mM) (compared to uninhibited control), on resistant *H. armigera* esterase activity (each track contains the equivalent of 0.3 mg tissue)

DISCUSSION

There is clear evidence that resistance to chlorfenapyr has occurred in Australian populations of *H. armigera* and this resistance is now reaching levels that may impact on the field performance of chlorfenapyr. A major cause of chlorfenapyr resistance is cross-resistance to an increasing pyrethroid resistance factor in *H. armigera* cotton populations and there continues to be extensive use of commercial pyrethroids (bifenthrin, αcypermethrin, β-cypermethrin, β-cyfluthrin, es-fenvalerate etc) against *H. armigera*. While cross-resistance obviously is a major cause of chlorfenapyr resistance, field use of chlorfenapyr may also have contributed to the resistance problem. Although current resistance management recommendations limit chlorfenapyr use to no more than two full rate sprays per season, chlorfenapyr is sometimes used at half rates and as band sprays, thereby increasing the actual number of chlorfenapyr spray events.

Resistance mechanism studies also support the link between chlorfenapyr and pyrethroid resistance. Our data clearly demonstrated that the esterases that sequester and hydrolyse pyrethroids, also bind to chlorfenapyr in *H. armigera*. While chlorfenapyr is not an ester and cannot be acting as a conventional esterase inhibitor, our observations suggest that chlorfenapyr is entering a physicochemical reaction with esterase, thus making it unavailable to the substrate. *H. armigera* esterase has a considerable sequestering ability towards pyrethroids (Gunning et al., 1996) and pyrethroid resistant *H. armigera* have a greatly increased production of esterase. Thus, highly pyrethroid resistant *H. armigera* could also sequester considerable quantities of chlorfenapyr and cause resistance. Esterase titre is also directly related to pyrethroid resistance factor (Gunning et al 1996) and hence the link of chlorfenapyr resistance to increasing levels of pyrethroid resistance.

In Australian cotton, it is essential to manage insecticide use to maintain efficacy of insecticides such as chlorfenapyr against *H. armigera*. Given the risk of the development of resistance to transgenic cotton, compounds, such as chlorfenapyr are very important to the Australian cotton industry. The Australian *Helicoverpa* resistance management currently restricts chlorfenapyr use to mid and late summer which coincides to a large degree with pyrethroid use. Given the present findings that high levels of pyrethroid resistance confer cross-resistance to chlorfenapyr, it would be advantageous, if chlorfenapyr were to be used earlier in the cotton season, so as not to overlap with the periods of highest pyrethroid resistance.

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Use of novel substrates to characterise esteratic cleavage of pyrethroids

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ABSTRACT

A rapid technique for determining the ability of resistance-associated esterases in pest insect species to hydrolyse pyrethroid esters is described. This assay has been applied to insecticide-resistant populations of peach-potato aphid (*Myzus persicae*), cotton aphid *(Aphis gossypii*) and cotton bollworm (*Helicoverpa armigera*).

INTRODUCTION

Biochemical tests for characterising specific resistance mechanisms provide a powerful means of high-throughput diagnosis to implement tactics for delaying or overcoming resistance. Such assays are routinely used for characterising acetylcholinesterase insensitivity in individual insects, but metabolic resistance mechanisms usually depend upon radiolabelled metabolite studies, or the use of a model substrate that may or may not mirror the metabolism of the insecticide.

We report here on the use of a novel substrate, 1-naphthyl-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate, to allow greater insight into the ability of insect esterases to hydrolyse pyrethroid esters. The validity of the substrate was confirmed using resistant *Myzus persicae* and purified esterase E4, for which the metabolism of permethrin isomers is known through previous metabolite studies (Devonshire & Moores, 1982).

MATERIALS AND METHODS

Insects

The origins and rearing of the *Myzus persicae* clones used in this study (US1L, laboratory susceptible and 800F, highly resistant) have been described elsewhere (Devonshire *et al*, 1983). The *Helicoverpa armigera* were 3rd instar larvae reared on artificial diet using a photoperiod regime of 12:12 hr. light:dark, temperature 27 °C. Strains used were a laboratory susceptible strain, German, obtained from Bayer (originally from the Ivory Coast) and Sirsa, a pyrethroid-resistant strain from India. An Australian strain (FenR) was collected from the field and shown to be pyrethroid-resistant after selection by fenvalerate (Gunning *et al*, 1996).

Synthesis of substrates

The 1-naphthyl esters (Fig. 1) of the four carboxylic acids, 1-(S) *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid, 1-(R) *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid, 1-(S) *cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid and 1-(R) *cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid, were prepared individually from the enantiomeric carboxylic acids which had been assessed as reported by Elliot *et al*, (1976). The esters were formed from the carboxylic acid (1eq.) and 1-naphthol (1.0 eq) using dicyclohexylcarbodiimide (2.2 eq.) and 4-N, N-dimethylaminopyridine (0.05 eq) in dichloromethane (Steglich & Neises, 1978). The esters were purified by silica gel dry column chromatography using a gradient of petroleum ether (40-60) and diethyl ether (100:0 to 80:20). The ^{1}H-nmr spectral data (400MHz, $CDCl_3$) agreed with the literature data for the racemic esters (Butte & Kemper, 1999).

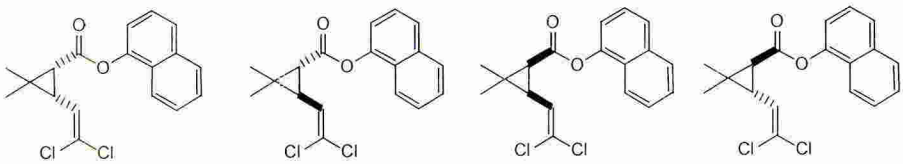

Fig 1. The four isomers of 1-naphthyl-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate

Assay procedure

The assay procedure is based on the spectrophotometric assay used for testing pyrethroid-cleaving enzymes in human serum (Butte & Kemper, 1999). Esterase activity was measured using 1-naphthyl-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate as the substrate. The released 1-naphthol was detected by reaction with Fast Blue RR Salt (Grant *et al*, 1989). For preliminary investigations, 20 *Myzus persicae* individuals were homogenised in 100µl of phosphate/ Triton (0.02M phosphate buffer, pH 7.0 containing 0.1% Triton X-100). 20µl aliquots were added to wells of a microplate containing 230 ul of Fast Blue RR Salt in phosphate buffer (0.2M, pH 6.0 containing 0.1% Triton X-100) containing substrate to give final assay concentrations of 100uM 1-naphthyl-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate and 0.7 mM fast Blue RR Salt. For single insect assays, individuals were placed in separate wells of a 96-well microplate (NUNC) containing 20µl of buffer (phosphate, pH 7.0 as above) and homogenised simultaneously using a multi-homogeniser designed for microtitre plates. Substrate and Fast Blue RR were added as above.

Assays were performed on a "Tmax" kinetic microplate reader (Molecular Devices), utilising the SOFTmax software package. Absorbance readings at 450 nm were taken every 20 secs for 1 hr.

Electrophoresis

Electrophoresis of individual *H. armigera* larvae was carried out on PAGE gels at 250V for 2 hrs. The details of the running system are those described by Gunning *et al*, (1996).

RESULTS AND DISCUSSION

In preliminary experiments, the four isomers of 1-naphthyl-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate were assayed against mass homogenates of susceptible and resistant *Myzus persicae* (Fig. 2.) It is clearly seen that the (1S)-*trans* isomer behaves differently to the three other isomers, as exemplified by (1S)-*cis*. After an initial 'burst' shown by all isomers, the (1S)-*trans* isomer exhibited a significant rate of hydrolysis . This is in keeping with earlier research which demonstrated that E4, the esterase responsible for conferring resistance in *Myzus persicae*, hydrolysed only (1S)-*trans* permethrin (Devonshire & Moores, 1982).

Both the initial 'burst' and the residual activity, which is actual hydrolysis of the pyrethroid-like substrate, were proportional to the amount of homogenate added. When an equivalent amount (in activity units) of purified E4 was added, a similar hydrolysis rate was observed without the initial 'burst' (Fig. 2).

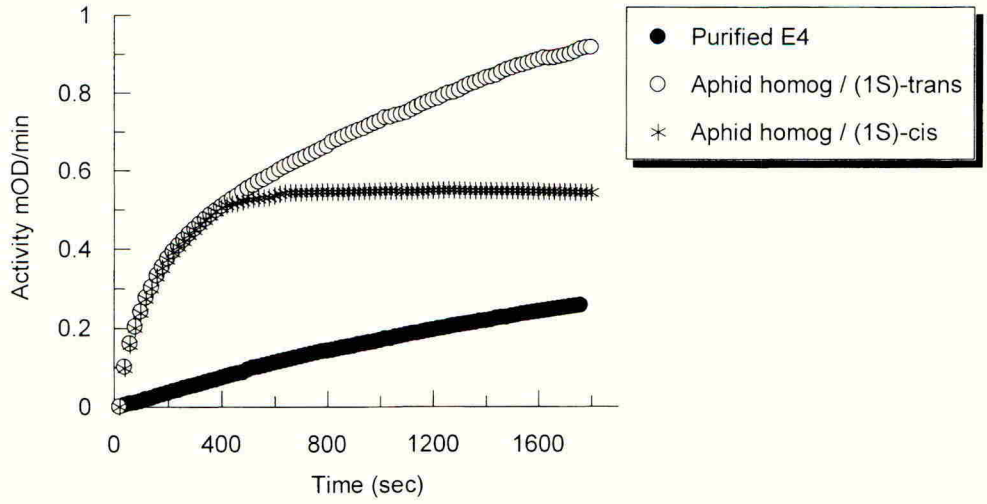

Fig. 2 Plot of activity at λ 450nm, illustrating the initial 'burst' of activity in aphid homogenates compared with purified esterase E4 and the hydrolysis of (1S)-*trans* compared with hydrolysis of (1S)-*cis*.

The initial 'burst' of high activity observed in these assays was also observed when assaying individual aphids (*Myzus persicae* and *Aphis gossypii*), but this phenomenon was not observed with *Helicoverpa armigera*. It is thought probable that naturally occurring phenols in the diet of the aphids were responsible for this, as the initial 'burst' was observed even in the absence of substrate, i.e. as would occur when endogenous phenols present in the insects reacted with Fast Blue RR. This initial complex was also a different colour (red/brown) than that produced by the reaction of 1-naphthol and Fast Blue RR Salt (yellow/green), corroborating this theory. Because the *H. armigera* larvae were reared on artificial diet, the plant phenols were not present.

Having optimised the conditions for the assay, individual *Myzus persicae* susceptible and resistant to insecticides were tested. A lag phase (5 mins) was introduced to remove the initial points of the assay because of the aforementioned initial 'burst'. Clear differentiation was observed between individual susceptible and resistant aphids. Using slopes calculated by SOFTmax (linear regression) a bi-variate plot of (1S)-*trans* hydrolysis was plotted against 1-naphthyl acetate hydrolysis (Fig 3). It is clearly seen that resistant individuals hydrolyse both substrates faster than susceptible individuals.

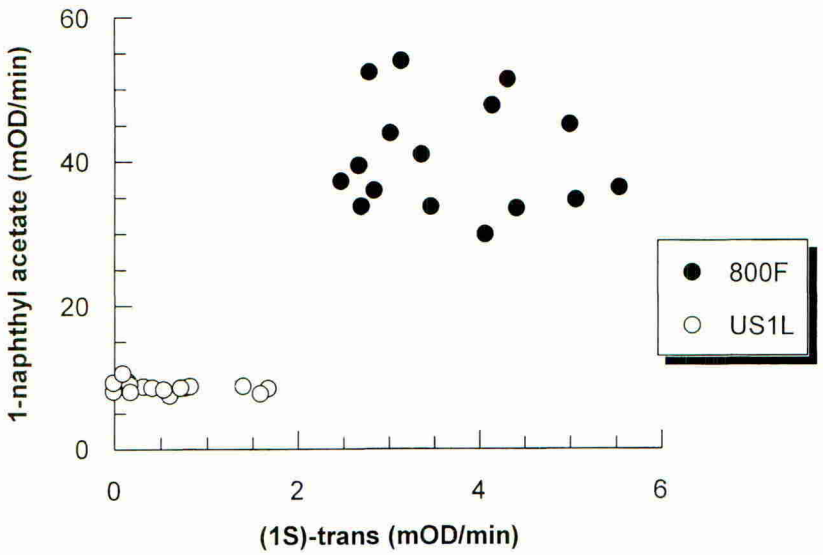

Fig 3. Bivariate plot showing rate of hydrolysis of 1-naphthyl acetate and (1S)-*trans* by the same individual aphid homogenate.

When the assay was applied to individual *H. armigera* larvae, it was found that the Oxford strain (low resistance to pyrethroids) showed significantly higher hydrolysis of the (1S)-*trans* isomer than individuals of the German (susceptible) strain (Fig. 4).

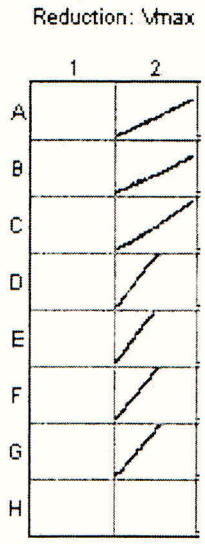

Fig 4. Individual *H. armigera* larvae assayed in a 96-well microplate. Wells 2A-2C are German strain, 2D-2G Oxford strain. 2H is a control blank.

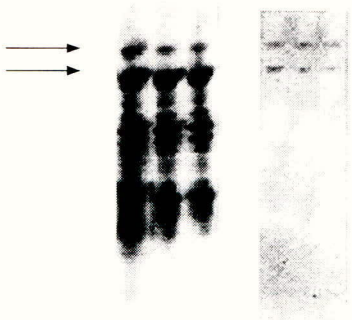

Fig. 5 PAGE gel of Australian pyrethroid-resistant *H. armigera* larvae stained using 1-naphthyl acetate (A) and the (1S)-*trans* isomer (B).

It is envisaged that this assay can be used with individuals of many arthropod species that exhibit pyrethroid resistance, and will be a powerful tool for characterising the pyrethroid-cleaving ability of the pests' esterases. However, it must be remembered that hydrolysis is not the only mechanism by which esterases can confer pyrethroid resistance. A PAGE gel stained using both 1-naphthyl acetate and the (1S)-*trans* isomer (Fig. 5) demonstrated that two slow-moving esterases (arrowed) hydrolysed the pyrethroid analogue. The main resistance-associated esterases are, however, the faster-running group of dark staining esterase bands (Gunning et al, 1996). This indicated that because the mechanism is one of sequestration, the novel substrate remained uncleaved.

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The effect of dose rate of imidacloprid and clothianidin on insecticide-resistant clones of *Myzus persicae* (Sulzer)

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ABSTRACT

In a field trial at Broom's Barn in 2001, the efficacy of imidacloprid (at 90 ,45 and 15 g a.i./unit) and clothianidin (at 60, 30 and 10 g a.i./unit) applied as coatings to pelleted seed was tested against clones of aphids with different combinations of resistance mechanisms. Both insecticides at all rates tested gave significant reductions in the number of all clones of aphid 46 and 53 days after sowing. The lowest rates of both insecticides gave significantly poorer control than the higher rates, but there were no differences between treatments, nor were there any interactions between treatments, rates and clones. So far therefore, there is no suggestion that imidacloprid or clothianidin will fail to control clones with moderate tolerance levels similar to those tested in this study.

INTRODUCTION

The peach-potato aphid, *Myzus persicae* acts as a vector of virus yellow disease, which, when transmitted to the sugar beet crop, can reduce yields by 50 %, making it the most important pest of sugar beet. Over time *M. persicae* has developed resistance to many insecticides, including organophosphates, carbamates and pyrethroids. Three resistance mechanisms have been identified in the U.K. The first discovered over thirty years ago is linked to the overproduction of E4 and FE4 (carboxylesterase enzymes). These enzymes inactivate organophosphate and carbamate insecticides before they reach the insect's nervous system. The levels of carboxylesterase (shown in laboratory bioassay studies) can be classified into one of four categories: S – susceptible, R_1 - moderately resistant, R_2 – highly resistant and finally R_3 – extremely resistant (Devonshire *et al.*, 1976). The second mechanism, modified acetylcholinesterase, commonly known as MACE, was first seen in the UK in 1995 (Moores *et al.*, 1994). With this mechanism, the insecticides' target enzyme, acetylcholinesterase, is altered or modified, rendering it immune from attack by the dimethyl-carbamates, pirimicarb or triazamate. MACE aphids caused serious problems on potatoes in Lincolnshire in 1996 (Foster *et al.*, 1998). More recently, a third resistant mechanism has been discovered, known as knock-down resistance (kdr). With this mechanism the sodium channel enzyme, which is responsible for the maintenance of nerve impulses down the length of a nerve cell, has been modified, such that it is immune to attack by pyrethroids (Martinez-Torres *et al.*, 1999). The three fore-mentioned resistance mechanisms act independently of one another, although there are some aphids that contain two, and some that contain all three. Aphids with any or all three mechanisms are well

controlled by the neonicotinoid insecticide, imidacloprid (Dewar *et al.*, 1998; Foster et al., in press).

However, clones of *M. persicae* have been discovered from field crops in the UK and other countries such as Greece that show some tolerance to imidacloprid (tolerance factor (TF) range 3-18 fold in relation to a susceptible standard) (Foster *et al.*, in press). In this paper we describe the results from a field trial on sugar beet, in which UK clones of *M. persicae* with different combinations of the above resistance mechanisms, with or without some degree of imidacloprid tolerance were exposed to imidacloprid and another novel neonicotinoid, clothianidin, at a wide range of doses.

MATERIALS AND METHODS

Trial design

The field trial was carried out in 2001 at Broom's Barn Experimental Station in Suffolk. The trial was deliberately sown late (24 May) to provide young plants during the early part of July. Ten treatments were randomised in four blocks (although this paper only shows results for seven of the treatments), giving a total of 40 plots. Each plot was 12m long and 3m wide (6 rows of sugar beet). Within each plot there were five sub-plots each containing a 1 x 1m field cage which covered two rows of beet plants. The cages, while not completely insect-proof, served to discourage colonisation by "wild" aphids prior to inoculation with the test clones and also to limit predation by predators such as ladybirds, and lacewing and syrphid larvae. Plant numbers under each cage were reduced to three. Because of a poor seed bed at drilling there was a difference in the rate of growth and leaf stage of the sugar beet, which varied between 2-4 and 8-10 leaves when they were inoculated on the 29th of June.

Aphid clones

Within each cage, each plant was inoculated with *circa* ten wingless adult aphids from one of five clones of aphids to be assessed, all of which had been reared on *Capsella bursa-pastoris* (Shepherd's purse). Details of the clones are as follows:

clone: 405D	- R1 kds	non-MACE	nicS (TF=1)
clone: 2160D	- R1 kdr	non-MACE	nicS (TF=1)
clone: 4239D	- R1 kdr	MACE	nicT (TF= 4.5)
clone: 3104B	- R1 kdr	MACE	nicT (TF=5.7)
clone: 2169G	- R3 super kdr	non-MACE	nicS (TF=1)

Nic S denotes susceptible to neonicotinoids, and nic T denotes tolerance. All clones were collected in England. 405D was collected from potatoes in Cambridgeshire in 1977, 2160D from Brussels sprouts also in Cambridgeshire in 1997, 4239D from glasshouse peppers in Suffolk in 2000, 3104B from oilseed rape in Cambridgeshire in 1998, and 2169G from Brussels sprouts in Lincolnshire in 1997.

The colonies were allowed to build up for seven days before the first of two samples was taken. One plant from each sub-plot was cut at the crown, placed in a sealed box and subsequently examined for aphids in the laboratory. Each clone was collected separately to

minimise opportunities for contamination. Adult or fourth instar aphids surviving treatments were placed in wells in ELISA plates and subsequently tested to confirm their esterase and MACE resistance status. Diagnostic bioassays were used to confirm kdr and imidacloprid status.

Treatments

Imidacloprid was applied to pelleted seed at full rate (90g) half rate (45 g) and one sixth rate (15 g) a.i./unit (one unit = 100000 seeds) by Germain's of King's Lynn in a process similar to their commercial application. Clothianidin was applied at the proposed commercial rate (60 g), half that (30 g), and one-sixth that rate (10g) a.i./unit. The lower rates were designed to mimic the levels of these insecticides in plants as they matured.

RESULTS

The aphids increased very rapidly in the warm conditions prevailing before assessments were made. Numbers in untreated plots ranged from 87 to over 280 per plant in the different clones. Forty six days after sowing there were no significant differences between clones but there were between treatments. All rates of both imidacloprid and clothianidin gave significant control of aphids, although the two higher rates of imidacloprid and the highest rate of clothianidin performed significantly better than the lower rates (Table 1). There was no difference between the highest rates of these two insecticides. There was no significant interaction between clones and treatments.

Fifty three days after sowing, numbers of aphids in the untreated plots remained similar in two clones but had declined substantially in the other three clones (Table 2), probably as a result of increased predation from ladybirds, which had been attracted to the food source. There were again no significant differences between clones, but there were between treatments. All insecticide treatments significantly reduced aphid numbers in all clones. The two higher rates of both imidacloprid and clothianidin gave better control than the lowest rate of each insecticide, but there was no difference between the two.

DISCUSSION

As was seen in 1998, all clones of *M. persicae* were well controlled by the commercial rate of imidacloprid and the proposed commercial rate of clothianidin. The half rates of each insecticide also gave adequate control of all clones, but the lowest rates, one sixth of the full rate, performed significantly worse, whilst still reducing numbers compared to the untreated control. These lower rates simulate the rates likely to be experienced by aphids when feeding on plants that were about 9-10 weeks old, at the time when the efficacy of the commercial treatments would be wearing off. In most commercial crops this would also coincide with the changing physiology of the plants as the leaves switched from sources of nutrients to sinks, and the roots began to store sugar. At this point the plants tend to become unpalatable to green aphids (Kift *et al.*, 1997), and cause them to accumulate a black substance in their guts, which eventually results in starvation and death. Thus the decline of the insecticides is not as important as it would be if the plants were still vulnerable to colonisation. However, in other plant species, for example oilseed rape, plants remain palatable for much longer. A

substantial proportion of these would probably survive fairly well. Nevertheless, at least with the clones tested here, there was no suggestion that the two clones reported as being tolerant to imidacloprid were better able to survive these treatments than those that were regarded as susceptible. So as far as sugar beet is concerned, there is not yet concern about controlling *M. persicae* that have been reported as being imidacloprid–tolerant.

ACKNOWLEDGEMENTS

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Table 1 Effect of insecticide seed treatments on the number of green aphids (mostly *Myzus persicae*) per plant ($\log_{10}(n+1)$) on sugar beet at Broom's Barn, 46 days after sowing

Treatment	Rate g a.i./unit	Clone					Mean All clones
		405D	2160D	4239D	3104B	2169G	
		R1 kds Non-MACE Nic S	R1 kdr Non-MACE Nic S	R1 kdr MACE NicT	R1 kdr MACE Nic T	R3 kdr Non-MACE Nic S	
Untreated		2.458 (286.1)	2.292 (195.0)	2.237 (171.6)	1.945 (87.2)	2.379 (238.6)	2.262 (182.0) [a]
Imidacloprid	15	1.464 (28.1)	1.482 (29.4)	1.436 (26.3)	1.337 (20.7)	1.330 (20.4)	1.410 (24.7) [b]
Imidacloprid	60	0.615 (3.1)	0.870 (6.4)	0.659 (3.6)	0.809 (5.5)	0.878 (6.7)	0.766 (4.8) [d]
Imidacloprid	90	0.540 (2.5)	0.707 (4.1)	0.527 (2.4)	0.595 (2.9)	0.600 (3.0)	0.594 (2.9) [d]
Clothianidin	10	1.559 (35.3)	1.830 (66.7)	1.635 (42.2)	1.435 (26.2)	1.702 (49.4)	1.632 (41.9) [b]
Clothianidin	30	1.179 (14.1)	1.034 (9.8)	1.272 (17.7)	1.180 (14.2)	0.783 (5.1)	1.090 (11.3) [c]
Clothianidin	60	0.868 (6.4)	0.863 (6.3)	1.172 (13.9)	0.787 (5.1)	0.721 (4.3)	0.882 (6.6) [cd]
SED tmt (27 df)							0.1437
SED tmt/clone interaction (120 d.f)				0.2839			
LSD (5%)				NS			0.2948

Figures in parentheses are back-transformed minus 1. Treatments with different letters are significantly different at $P<0.05$;
NS = non significant;

Table 2 Effect of insecticide seed treatments on the number of green aphids (mostly *Myzus persicae*) per plant ($\log_{10}(n+1)$) on sugar beet at Broom's Barn, 53 days after sowing

Treatment	Rate g a.i./unit	Clone					Mean All clones
		405D	2160D	4239D	3104B	2169G	
		R1 kds Non-MACE Nic S	R1 kdr Non-MACE Nic S	R1 kdr MACE NicT	R1 kdr MACE Nic T	R3 kdr Non-MACE Nic S	
Untreated		1.700 (49.1)	2.297 (197.2)	2.227 (167.7)	1.561 (35.4)	2.087 (121.3)	1.975 (93.3) [a]
Imidacloprid	15	1.400 (24.1)	1.225 (15.8)	1.544 (34.0)	1.233 (16.1)	1.504 (30.9)	1.381 (23.0) [b]
Imidacloprid	60	0.683 (3.8)	0.639 (3.4)	0.362 (1.3)	0.537 (2.4)	0.676 (3.7)	0.579 (2.8) [c]
Imidacloprid	90	0.433 (1.7)	0.508 (2.2)	0.389 1.5)	0.512 (2.3)	0.314 (1.1)	0.431 (1.7) [c]
Clothianidin	10	1.341 (20.9)	1.571 (36.3)	1.605 (39.2)	1.414 (25.0)	1.545 (34.1)	1.495 (30.3) [b]
Clothianidin	30	1.110 (11.9)	0.679 (3.8)	0.301 (1.0)	0.584 (2.8)	0.926 (7.4)	0.720 (4.3) [c]
Clothianidin	60	0.301 (1.0)	0.540 (2.5)	0.278 (0.9)	0.413 (1.6)	0.226 (0.7)	0.352 (1.3) [c]
SED tmt (27 df)							0.1788
SED tmt/clone interaction (120 d.f)				0.3444			
LSD (5%)				NS			0.3669

Figures in parentheses are back-transformed minus 1. Treatments with different letters are significantly different at $P<0.05$;
NS = non significant;

Resistance to carbamate, organophosphate and pyrethroid insecticides in the potato aphid (*Macrosiphum euphorbiae*)

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ABSTRACT

A large number of *Macrosiphum euphorbiae* clones, initiated from field samples collected on potatoes in the UK since 1998, have produced cross-reactions in an immunoassay previously developed to assess levels of E4/FE4 carboxylesterase resistance in peach-potato aphids (*Myzus persicae*). Mean immunoassay values of each *M. euphorbiae* clone fell within the S-R_2 range used to categorise individual *M. persicae* (representing susceptible through to highly resistant forms). These values were associated with resistance of the *M. euphorbiae* clones to pirimicarb and lambda-cyhalothrin measured in laboratory leaf-dip bioassays. This suggests that this species has developed a metabolic resistance mechanism based on over-production of an esterase analogous to that in *M. persicae*. Furthermore, levels of esterase resistance appeared to be sufficient to confer selective advantages in the field as a substantial majority of the aphid samples taken from crops recently treated with insecticides contained high esterase forms compared with samples that had not received treatment. Biochemical studies indicated that resistance to dimethoate is primarily conferred by a mechanism based on AChE insensitivity (MACE).

INTRODUCTION

A number of aphid species are exposed regularly to insecticides in the UK. However, only a few of these have been reported to have evolved resistance, namely the peach-potato aphid, *Myzus persicae* (Foster *et al.*, 2000), the cotton aphid, *Aphis gossypii* (Gubran *et al.*, 1992; Suzuki & Hama, 1998), the damson-hop aphid, *Phorodon humuli* (Herdig, 1982) and the currant-lettuce aphid, *Nasonovia ribisnigri* (Barber *et al*, 1999). Some factors contributing to resistance development seem clear-cut, eg. virtual restriction to glasshouses in which resistance risks are inherently high (*A. gossypii*) or to single crop species that receive frequent treatment with insecticides (*P. humuli* and *N. ribisnigri*).

In contrast, *M. persicae* is a highly polyphagous species occurring in glasshouses and a succession of crop and non-crop hosts in the field. Its proneness to developing resistance must, to a large extent, reflect a complex interplay between the phenology and dispersal behaviour of the aphid, and the temporal sequence of insecticide treatments on crops it inhabits. In this context, however, it is notable that the potato aphid, *Macrosiphum euphorbiae*, despite having a superficially very similar ecology to *M. persicae*, has not previously been reported to show resistance. We report here on the first confirmation of resistance in *M. euphorbiae*, and comparison of cross-resistance patterns and possible mechanisms with *M. persicae*.

MATERIALS AND METHODS

Collection of *Macrosiphum euphorbiae* from field potatoes

42 live *M. euphorbiae* samples were taken from field potatoes between July 1998 and October 2001. In each sample, aphids were collected, along with their supporting leaves, from plants at scattered positions throughout the collection site. The samples were then immediately transported by post to Rothamsted in a staple-sealed plastic bag inside a robust box. Each sample was accompanied by a record of place and date of collection and, in most cases, recent insecticide treatment history. On arrival of each sample at Rothamsted, several live *M. euphorbiae* clones were established and 4th instar and adult aphids were sorted and placed individually into the wells of a 96-well micro-plate for biochemical testing.

Aphid leaf-dip bioassays

Adult alatae from each *M. euphorbiae* clone were assessed for survival on leaf discs dipped in aqueous solutions of either formulated pirimicarb (Aphox)), dimethoate (Danadim) or lambda-cyhalothrin (Hallmark) at a range of concentrations. Control discs were dipped in water only. Aphid response to pirimicarb, dimethoate and lambda-cyhalothrin was then assessed after 24, 48 and 72 h respectively. Aphids were scored as affected if they had died or showed poor co-ordination. EC_{50} values were calculated by probit analysis using the POLO programme (Leora software, 1987). Results presented for each compound are based on pooled data from three to five separate bioassays each containing up to 180 aphids per clone. Six *M. euphorbiae* clones were assessed (Table 1).

Biochemical assays for esterase and MACE activity

M. euphorbiae that had been collected from the field or taken from laboratory lines were tested in an immunoassay to determine how much carboxylesterase they contained (Devonshire and Moores, 1982). The results were presented as an optical density reading for each aphid, indicating the level of enzyme activity. Testing for MACE activity was done using a kinetic enzyme assay in the absence and presence of diagnostic concentrations of pirimicarb and dimethoate (Moores *et al.*, 1994).

RESULTS

Aphid leaf-dip bioassays

Table I summarises the resistance factors shown by the *M. euphorbiae* clones to pirimicarb, dimethoate and lambda-cyhalothrin. These ranged up to 13, 57 and 5 respectively relative to the susceptible standard clone (4086C). There were significant positive associations between mean clonal $\log_{10}$ esterase activity in the immunoassay (measured at the time when the insecticide bioassays were done) and $\log_{10}$ EC_{50}'s to pirimicarb (linear regression: $F_{2,4} = 39.7$, $P = 0.002$) and lambda-cyhalothrin (linear regression: $F_{2,4} = 12.3$, $P = 0.02$). However no significant association was seen for the equivalent comparison using dimethoate (linear regression: $F_{2,2} = 7.91$, $P = 0.11$) although it should be noted that this was based on a smaller number of clones ($n = 4$).

Table 1. Response of *Macrosiphum euphorbiae* clones in leaf-dip bioassays applying pirimicarb, dimethoate and lambda-cyhalothrin.

Clone	Esterase category	EC_{50}[1] (ppm)	95% CI[2]	Slope	RF[3]
		PIRIMICARB (di-methylcarbamate)			
4086C	low	3.37	2.72-4.15[a]	4.4	**1.0**
2849C	low	6.50	4.81-8.45[b]	4.9	**1.9**
4368B	medium	8.58	7.54-9.96[b]	5.5	**2.9**
4353P	high	17.5	14.3-21.7[c]	6.8	**5.9**
4030A	high	23.0	17.4-30.9[c]	5.3	**6.8**
2783A	high	43.9	35.5-56.5[d]	3.2	**13**
		DIMETHOATE (organophosphate)			
4086C	low	0.25	0.19-0.31[a]	5.4	**1.0**
4030A	high	0.74	0.58-1.04[b]	7.4	**3.0**
2783A	high	1.77	1.48-2.09[c]	3.1	**7.1**
4353P	high	2.57	2.31-2.84[d]	5.6	**10**
		LAMBDA-CYHALOTHRIN (pyrethroid)			
4086C	low	0.06	0.03-0.10[a]	2.3	**1.0**
2849C	low	0.07	0.05-0.09[a]	2.8	**1.1**
2783A	high	0.14	0.10-0.17[ab]	3.2	**2.3**
4368B	medium	0.20	0.15-0.26[bc]	7.0	**3.2**
4030A	high	0.30	0.25-0.38[c]	2.2	**5.0**
4353P	high	0.31	0.20-0.48[c]	2.8	**5.0**

[1]Effective concentration resulting in 50% dead or poorly co-ordinated aphids;
[2]95% confidence limits; values followed by the same letter do not differ significantly;
[3]Resistance factor = EC_{50} for clone/clone 4086C.

Biochemical assays for esterase and MACE activity

Overall, 34% of the *M. euphorbiae* samples contained aphids with high levels of esterase (based on the categorisation system: low esterase < -0.85; medium esterase -0.85 to –0.22; high esterase > -0.22 $\log_{10}$ esterase activity in the immunoassay) (Fig 1). Indeed, the frequency of these forms appears to have remained relatively stable from year to year (1998: 2 of 3 = 67%; 1999: 7 of 21 = 33%; 2000: 1 of 4 = 25%; 2001: 5 of 14 = 36%). Although no AChE insensitivity (MACE) to pirimicarb was detected, there were indications of dimethoate insensitivity in several *M. euphorbiae* clones including all three high esterase forms assessed in the dimethoate leaf-dip bioassays.

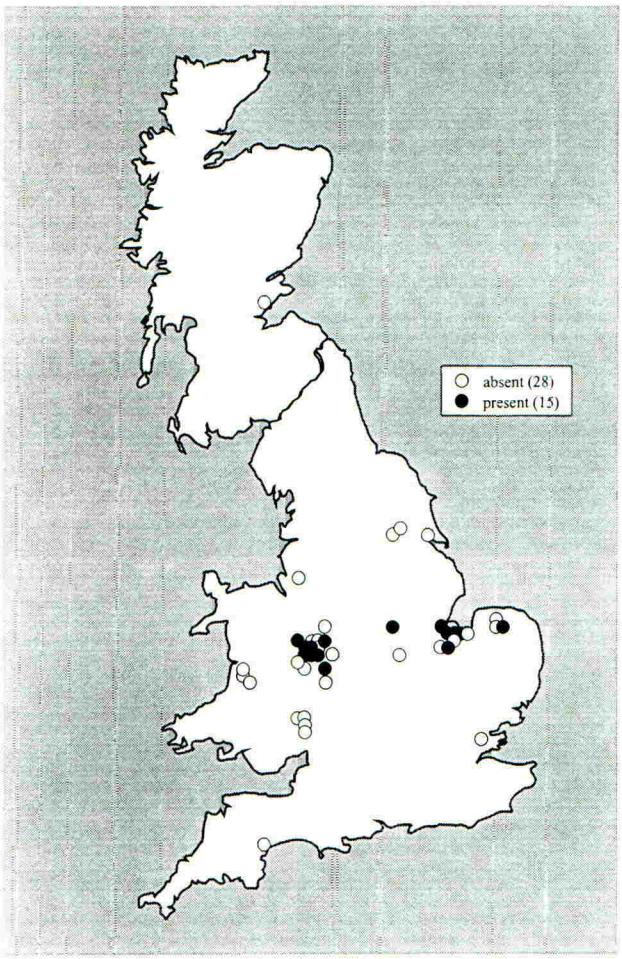

Figure 1. Presence of high esterase forms in *Macrosiphum euphorbiae* samples collected from UK field potatoes between 1998 and 2001.

Resistance to insecticides in the field

Taking the aphid samples that were accompanied by treatment records, all seven that had received applications of insecticides (either pirimicarb, aldicarb, oxamyl and/or lambda-cyhalothrin) within the last 10 days contained aphids scoring within the high esterase category. However, only six of the 23 samples that had not received any recent treatment contained these forms (Goodness of fit Chi- square + Yates' correction = 9.12, $P < 0.005$) (Table 2).

Table 2. Number of *Macrosiphum euphorbiae* field samples with and without high esterase forms collected from untreated potatoes or potatoes treated with insecticides.

Treated samples		Untreated samples	
Absent	Present	Absent	Present
0 (0%)	7 (100%)	17 (74%)	6 (26%)

DISCUSSION

The variability that exists in the esterase activity of *M. euphorbiae* collected from UK field potatoes, measured in an immunoassay originally developed for *M. persicae*, appears to be associated with resistance in laboratory bioassays to pirimicarb (a carbamate) and lambda-cyhalothrin (a pyrethroid). Further biochemical studies are needed to establish whether resistance is conferred by over-production of a specific esterase. Indeed, the cross-reaction in the specific immunoassay used may be due to the enzymes involved being closely related in both species. However, this cannot be confirmed until the relevant esterase is purified in *M. euphorbiae*. Having said this, the resistance factors to the two insecticides appear similar to those gained previously for the esterase mechanism in *M. persicae* suggesting at least some factors in common. Esterase activity in *M. euphorbiae* did not appear to be associated with resistance to dimethoate (an organophosphate). However, kinetic studies suggest that resistance in this case may be primarily conferred by a MACE mechanism.

To our knowledge, no apparent practical resistance, capable of compromising insecticide treatments at recommended rates at the time of application, has been reported for *M. euphorbiae* in the field in the UK. However, a significant proportion of our field samples that had received recent treatments with insecticides (primarily carbamates) contained aphids with high levels of esterase compared with untreated samples. This implies the existence of selection favouring these forms when insecticides are present at concentrations lower than those expected at the time that correct treatments are applied. Such conditions can arise from the natural decay of insecticides with time, or the application of reduced rates, either deliberately to cut costs or to target pests other than aphids. It is not unreasonable to believe that these 'windows of selection' represent environments that will favour the evolution of greater resistance in *M. euphorbiae* in the future.

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Resistance to insecticides in the currant-lettuce aphid, *Nasonovia ribisnigri*: laboratory and field evidence

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ABSTRACT

Bioassays conducted on twelve UK field populations of *Nasonovia ribisnigri* between 1999 and 2001 showed low-level resistance to pirimicarb (up to a maximum of *c.* 5-fold) and higher resistance (*c.* 25-fold) to pyrethroids. While there was consistent cross-resistance between the three pyrethroids tested, the mechanism of resistance appeared to be different from that for pirimicarb. Responses to pirimicarb were positively associated with non-specific esterase activity. Despite the low-level resistance seen in bioassays, there was a decreased effective period of pirimicarb in field experiments against pirimicarb-resistant aphids compared to the susceptible control, whereas a pyrethroid resistant clone demonstrated increased survival immediately after treatment with pyrethroids in field trials compared to the control.

INTRODUCTION

Nasonovia ribisnigri is the only one of the three leaf colonising species of aphid attacking lettuce foliage in the UK that is a specialist on this crop (Blackman & Eastop, 1984). Unlike the other two species, *Myzus persicae* and *Macrosiphum euphorbiae*, it favours feeding in the centre of the plant, forming large colonies in the lettuce heart that can be difficult to control with contact insecticides in the advanced stages of crop development. Unlike *M. persicae* it is not known as a virus vector but is a significant problem as a contaminant of lettuce heads that leads to rejection of produce from processing. It is a major pest of lettuce across Europe (Reinink and Dieleman, 1993) and has recently spread to America and New Zealand.

Control of this aphid in the UK relies mainly upon carbamate and pyrethroid insecticides as well as imidacloprid treated seed. Published bioassay data has shown resistance in UK field populations to pirimicarb (up to *c.* 19-fold) and cypermethrin (up to *c.* 4-fold) (Barber *et al.*, 1999). In these populations, pirimicarb resistance was associated with an elevated esterase (E0.34) as visualised on a polyacrylamide gel.

In this paper, we update the resistance status of *N. ribisnigri* in the UK and demonstrate the impact of resistance on field control.

MATERIALS AND METHODS

Aphid strains and rearing methods

The three laboratory 'standard' clones maintained in culture were: Nr1A, a fully susceptible strain; Nr2A, collected in 1997 from a site in Kent experiencing control problems with pirimicarb (Barber *et al.*, 1999); and Nr4A, collected from a site on the south coast experiencing problems with pyrethroids. In addition eleven uncloned field strains collected between 1999 and 2001 from the north (5 strains), south (1 strain), east (4 strains) and west (1 strain) of England were examined. Field experiments were conducted using the Nr1A (susceptible), Nr2A (pirimicarb resistant) and Nr4A (pyrethroid resistant) clones.

All strains of *N. ribisnigri* were reared parthenogenetically in the laboratory on whole plants of *Lactuca sativa* cv. 'Webb's Wonderful', without exposure to insecticides, at 21°C with a 16:8h (L:D) photoperiod. Plants were changed regularly and new ones re-infested to avoid host plant deterioration and excessive crowding of aphids. Prior to field trials, clones were maintained in glasshouses on *L. sativa* cv. Saladin.

Insecticides

Formulated insecticides used for leaf-dip bioassays were cypermethrin ('Cythrin', 100g/l EC); deltamethrin ('Decis', 25g/l EC); lambda-cyhalothrin ('Hallmark', 50g/l EC) and pirimicarb ('Aphox', 500g/kg SG). For leaf-dipping, all formulations were diluted to the required concentration in distilled water containing 0.01% 'Agral' (Zeneca Agrochemicals), a non-ionic surfactant added to improve leaf-wetting and to compensate for the loss of formulant at low insecticide concentrations.

Formulated insecticides used for field experiments were pirimicarb ('Pirimor') at a rate of 0.5g/l at 600l/ha and deltamethrin ('Decis') at a rate of 250ml/ha at 600l/ha, applied by hand held 1.5m boon at a height of 1m above the crop.

Bioassays

Leaf discs (35mm diameter) cut from lettuce (*L. sativa* cv. 'Webb's Wonderful') were dipped in insecticide solution for 20s, placed upside down on an agar bed (25mm in depth) in disposable plastic containers (30mm high), and allowed to air-dry. Alate adult *N. ribisnigri* of the required strain (10 per container) were placed on the treated leaf surface and confined by applying a ring of fluon to the exposed lip of the container. Leaf discs dipped in water plus Agral were used as controls. Bioassay containers were covered with a fine mesh lid and stored upright in a constant environment facility at 20°C under ambient daylight conditions.

Dose-response bioassays against the field populations were tested at least once over 3 - 5 concentrations with three batches of 10 alate adults per concentration. Adults incapable of co-ordinated movement of legs (after gentle prodding if necessary) were scored as dead. All bioassays were scored after 72h following initial exposure to insecticide. Owing to the low number of insects and the possibility of genetic heterogeneity within strains, no attempt was made to fit probit lines to these data. Association between responses to the four insecticides were investigated by pairwise comparisons of mortality data at concentrations yielding the

widest range of responses to each individual insecticide (i.e. those of greatest diagnostic value).

Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoretic patterns of non-specific esterases in individual aphids after homogenisation in sucrose/Triton X-100 (5%/1.6%) were analysed using 7.5% polyacrylamide gel slabs containing 0.2% Triton X-100 and a discontinuous buffer system (Davis, 1964) run at 250V for 2h. Gels were rinsed in 0.2M phosphate buffer, pH 6.0 for 30min then stained in the same buffer containing 5mM Fast Blue RR, 1% acetone and 0.6mM 1-naphthyl acetate. Gels were fixed and stored in 7% acetic acid.

Field experiments

The field experiment tested the effectiveness of pirimicarb and deltamethrin for controlling three clones of *N. ribisnigri* (Nr1A, Nr2A and Nr4A) as compared to untreated control plants. Lettuce plants (cv. Saladin) were grown for three weeks in an insect proof cage in a glasshouse until planting on 9 May 2000 into individual plots. Each plot was planted with two rows of ten plants. Plant spacing was 30cm within rows and 45cm between rows. Each plot of 20 plants was covered with an insect proof mesh cage the following day. Each of the nine treatment combinations (three aphid clones X three treatments) were assigned randomly to a single plot in each of six blocks, giving six replicates of each of the nine treatment combinations (54 plots in total).

Once established, each lettuce plant was inoculated with aphids of the appropriate clone by placing a piece of leaf from the bulked-up aphid cultures containing approximately ten aphids in the middle of each plant. Owing to poor weather, inoculated *N. ribisnigri* were allowed to establish for 17 days before the first pre-treatment sample was taken. The following day plants were treated with either, pirimicarb (0.5g/l at 600l/ha) or deltamethrin (250ml/ha at 600l/ha) or left untreated (control). Plants were then sampled two and six days after treatment. Of the 20 plants in each cage, six were cut, individually bagged and taken to the laboratory on each sampling occasion. The total number of small nymphs per plant was recorded.

Aphid numbers per plot (summed across the six sampled plants per occasion) were analysed using a generalised linear model framework, using a log-linear model assuming Poisson distributed data. Over-dispersion of the counts was allowed for in the analysis. Analyses included the total pre-treatment count (after $\log_e$ transformation) as a covariate to adjust for plot differences in the numbers prior to treatment. Treatment effects estimated in the model are re-expressed in terms of the percentage mortality for each chemical treatment relative to the untreated control.

RESULTS

Bioassays

Leaf-dip bioassays showed a wide range of resistance levels by field populations to all four compounds tested. Figures 1a-c show a close association of responses (i.e. cross-resistance)

to all three pyrethroids, whereas figure 1d shows no correlation between pyrethroid and pirimicarb resistance ($P<0.05$).

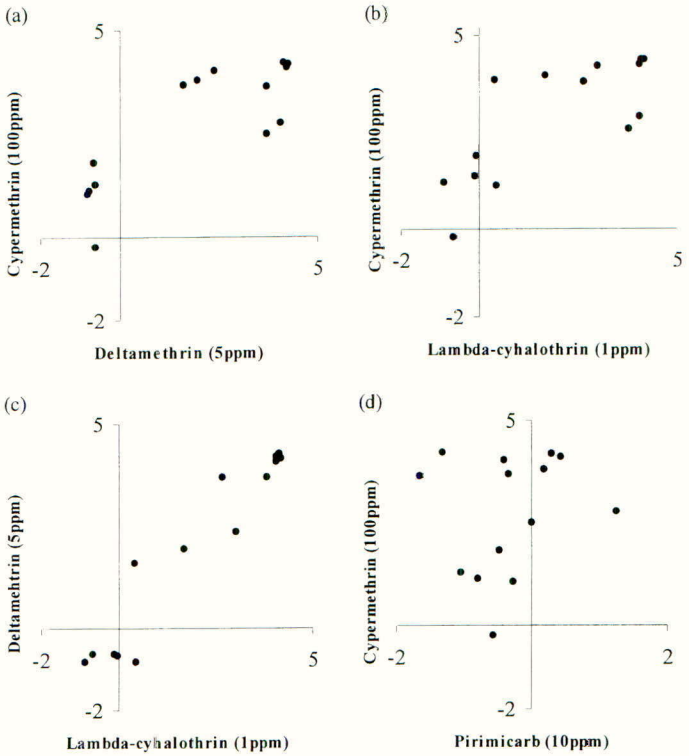

Figure 1a-d. Bi-variate plots showing responses of 14 *Nasonovia ribisnigri* populations to four different insecticides after 72h. Axes show logit transformed mortality data.

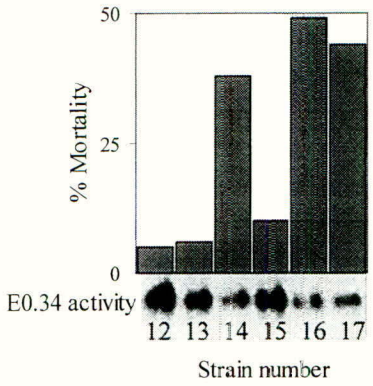

Figure 2. Comparison of E0.34 stain intensity in a selection of *Nasonovia ribisnigri* field populations and response to a single concentration (10ppm) of pirimicarb at after 72h.

Electrophoresis

Figure 2 shows the close association between the activity of the *N. ribisnigri* esterase, E0.34, previously described by Barber *et al.* (1999) and response to pirimicarb in bioassays. Of the populations samples shown, those demonstrating low mortality at 10ppm pirimicarb after 72h had high esterase activity at the position of E0.34 and *vice versa.*

Field experiment

Aphid mortality (restricted here to data for young nymphs) on pirimicarb and deltamethrin treated plants is summarised in figure 3a-b. There was no difference in the number of young nymphs between the susceptible Nr1A clone and the pirimicarb resistant clone, Nr2A, on plants two days after treatment with pirimicarb. However, six days after treatment, mortality of small nymphs for Nr2A was significantly less than for Nr1A and Nr4A ($P < 0.1$). In contrast, there was a significant difference in mortality between clones immediately after deltamethrin treatment with the pyrethroid resistant clone, Nr4A, demonstrating significantly reduced mortality, both two and six days after treatment, compared to the other two clones ($P < 0.1$).

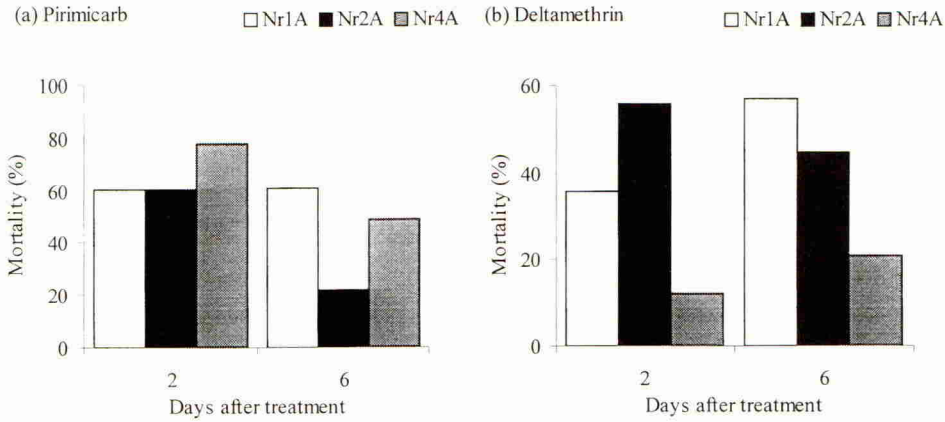

Figure 3a-b. Percent mortality of young nymphs two and six days after spraying, related to number on untreated plants. Populations were Nr1A (fully susceptible), Nr2A (pirimicarb resistant) and Nr4A (pyrethroid resistant).

DISCUSSION

Bioassays of strains of *N. ribisnigri* collected between 1999 and 2001 have generally supported the conclusions of (Barber et al., 1999) concerning the occurrence of significant differences in response to pirimicarb and pyrethroids in the UK. However, levels of pirimicarb resistance appear lower than that reported in the previous study, and resistance to

pyrethroids has, in a number of strains, tended to increase. Confirmation of apparent widespread cross-resistance between all three pyrethroids available for lettuce aphid control in the UK is of concern and highlights the need for very cautious use of this class of insecticides when targeting *N. ribisnigri* specifically. The mechanism of pyrethroid resistance is still uncertain, whereas recent work has continued to implicate enhanced activity of E0.34 in pirimicarb resistance both indirectly through correlations between esterase activity and bioassay data, and directly through enzyme binding studies (unpublished data). Pirimicarb resistance is still far lower than that reported for strains from southern France, in which it appears primarily attributable to reduced sensitivity to acetylcholinesterase, the target-site of carbamate insecticides (Rufingier *et al.*, 1999).

Despite the low levels of pirimicarb resistance recorded in laboratory bioassays, the field experiments showed that they did result in reduced control. The results suggest that pirimicarb resistance is expressed primarily in terms of a reduced period over which pirimicarb killed this clone effectively. Thus after comparable initial kill for pirimicarb-resistant and susceptible *N. ribisnigri,* surviving Nr2A reproduce earlier leading to a more rapid population recovery than susceptible *N. ribisnigri.* This contrasts with the effect of deltamethrin on pyrethroid-resistant (Nr4A) and pirimicarb-resistant (Nr2A) *N. ribisnigri.* In this case there was significantly lower initial mortality of Nr4A aphids compared to Nr2A.

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PCR-based method for detecting mutation allele frequencies for QoI resistance in *Plasmopara viticola*

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ABSTRACT

Field strains of *Plasmopara viticola*, the causal agent of grapevine downy mildew, were characterised for their sensitivity to QoI (Quinol outside Inhibitors) fungicides. At the gene level, resistance to this agricultural fungicide class is due to a Single Nucleotide Polymorphism on cytochrome bc1 mitochondrial gene causing an amino acid change (Glycine to Alanine at position 143 of the gene) in an enzyme of the respiratory chain. The mutation can be detected on mitochondrial DNA fragments with quantitative PCR amplification using allele specific primers and a Sybr Green detection method. A comparative analysis of this quantitative PCR measurement of mutant allele frequencies and the determination of biological phenotypes of several *P. viticola* samples has detected the presence of two characterised population classes: samples sensitive to QoI fungicides containing less than 0.1% of mutant allele and resistant ones containing more than 2%. The samples in which mutant allele percentage is between these two limits are mostly sensitive but, under the growth conditions used, they are able to become resistant and *vice versa*. Thus it seems that in a window between 0.1% to 2% of mutant allele present in the sample tested, the resistance of *P. viticola* to QoI fungicides is unstable.

INTRODUCTION

The class of Qo inhibitors (QoI) constitute a large group of fungicides used in agriculture including the strobilurines, fenamidone and famoxadone. These fungicides act as inhibitors of the cytochrome bc1 complex at the Qo centre in the respiratory chain of fungal mitochondria. The risk of QoI resistance development was anticipated prior to their commercial introduction because of their single site mode of action. Several point mutations in the *cytochrome b* gene have been identified, first in the yeast *Saccharomyces cerevisieae* (Di Rago, *et al.*, 1995) and then in several fungi. The particular mutation G143A (Glycine to Alanine at position 143 of the gene) has since been identified and characterised in subpopulations of pathogenic fungi resistant to QoIs under practical conditions of disease control in *Venturia inaequalis*, *Blumeria graminis*, *Pseudoperonospora cubensis* (Zheng, *et al.*, 2000; Sierotski, *et al.*, 2000; Ishii, *et al.*, 2001). The objective of this study was to analyse the *cytochrome b* gene of *Plasmopara viticola* (vine downy mildew) for the G143A point mutation and to correlate the presence of this mutation with the resistant phenotype.

MATERIALS AND METHODS

Sample collection and shipment

P. viticola populations were collected during the season of downy mildew (June-August 2001) in different vineyards. QoI treatments were included in the trials. The frequency of the application was limited by the maximum number of QoI applications recommended by the local authorities or by the FRAC QoI Working Group Guidelines. Five to ten leaves were harvested in each location, trial plot or field. This number is normally considered adequate and representative of one location. Leaves randomly collected showed distinct lesion development. Leaves were washed to remove product residue and collect a fist set of sporangia representative of that found in the field (P0 population). The washed leaves intact or limited to the lesion areas were then placed in sterile humid chambers at 17-20°C to promote sporulation and guarantee good viability of the samples. Sporangial populations were subcultured one or more times to obtain sufficient material to perform the biological test. Sporangial solution obtained after these subcultures (1 or 2 steps) is called P1 population and is enough concentrated to perform the resistance test.

Biological test procedure to identify the resistance phenotype

Fenamidone (SC 500 g/litre) was applied as a one day preventive spray using a sprayer calibrated to deliver the equivalent of 250 litre/ha to vine leaf discs supported on survival agar medium (10 g/litre), complemented with 2 mg/litre of kinetin, in Petri dishes. 24 to 30 discs were employed per rates in 2 or 3 Petri dishes, according to the number of sporangia available. Two discriminating dose rates were applied: 10 mg/litre (minimum inhibitory concentration) and 50 mg/litre. Inoculation was carried-on by depositing a 10µl droplet of sporangial suspension (20000 to 40000 spores/ml) on the adaxial face of each disc. The Petri dishes were then incubated under controlled conditions (18°C) using a 12h photoperiod. The Percentage sporulating area originating from the droplets was assessed 8 days after treatment and the phenotype defined as resistant when sporulation was observed and sensitive where no sporulation was detected.

Quantitative PCR test procedures

Two ml of sporangial suspension (20000 to 40000 spores/ml) obtained from *P. viticola* were centrifuged for 10 min at about 10000*g* in order to collect pelleted material. DNA was purified with the Qiaprep system (Qiagen) according to the manufacturer's protocol and without the buffer PB wash step. Two quantitative PCR tests were performed on each sample using a mutation specific primer: 5'-GGACAAATGAGTTTTTGGGG-3' for the wild type allele or 5'-GGACAAATGAGTTTTTGGGC-3' for the mutant allele and a common reverse primer: 5'-ATTATCAACGGCGAATCCAC-3'. The PCR reaction mixture contained 5µl of DNA extract, 0.3 µM of each primer, a final concentration of 5mM of $MgCl_2$ and Faststart DNA Master Sybr green I (Roche). PCR reactions were performed in a Lightcycler system (Roche) programmed for 10 minutes of enzyme activation (95°C), followed by 50 cycles of 10s at 95°C, 5s at 55°C, 6s at 72°C. To verify the purity of the product obtained, a melting curve was performed at the end of the PCR with an increase of the temperature of 0.05°C/s between 55 and 95°C. Quantitative PCR crossing points corresponding to quantity of the amplified gene were obtained with the second derivative maximum method. These were

named respectively Cpwt or Cpmut for the crossing point obtained with primers corresponding to the wild type or mutant allele. The ratio (r) of the 2 allele presence in samples was obtained using the delta Cp method. $r=(1+E)^{\wedge(Cpwt-Cpmut)}$ where E is the PCR efficacy normally equal to 1. The subsequent mutant allele percentage is $x=r/(r+1)$.

RESULTS

During the 2001 *P. viticola* season the study was performed on 266 samples collected in France, Italy and Spain either from Bayer Cropscience field trials or from fields of independent cultivators. It should be noted that this collection is not representative of the European QoI sensitivity situation: strains were, in the majority, collected to perform fungicide performance surveys in fields previously treated with QoI fungicides. Among these samples, 131 were determined to be resistant to the QoIs and 135 were sensitive.

One objective was to provide information at the biological molecular level on the above mentioned samples. Many publications have suggested that a single mutation in the cytochrome b site of some fungi is sufficient to cause QoI resistance. Protein sequence alignments were made between cytochrome b protein of known organism and partial protein sequence of *P. viticola*. Two *P. viticola* protein sequences were obtained by the translation of gene sequences coming from amplification. PCR was performed using primers designed on the known cytochrome b gene sequence of another Oomycete fungus: *P. infestans*. The two partially sequenced strains were biologically resistant or sensitive to QoI. The protein alignment shown in figure 1 clearly identifies the point mutation responsible for QoI resistance in *P. viticola*.

Mycena galopoda	FLGYVLPFGQMSLWAATV
Mycena viridimarginata	FLGYVLPFGQMSLWGATV
Saccharomyces cerevisieae	FLGYCCVYGQMSHWGATV
Phytophthora infestans	FMGYVLPWGQMSFWGATV
Plasmopara viticola (S)	FMGYVLPWGQMSFWGATV
Plasmopara viticola (R)	FMGYVLPWGQMSFWAATV
	129 146

Figure 1. Partial alignment (positions 129 to 146) of the deduced amino acid sequence of the cytochrome b gene fragment known to contribute to the Qo centre in different fungus. *Plasmopara viticola* (S) is a QoI sensitive strain and (R) is a QoI resistant (R). Site of amino acid substitution is framed.

The PCR amplification was performed on the same sample used for the resistance phenotype study, *i.e.* on P1 population. The mutation (G143A) can be detected on DNA from all resistant samples with quantitative PCR amplification using allele specific primers and a Sybr

Green detection method. Therefore, a new method to quantify the percentage of mutated alleles at the position 143 of the *cytochrome b* gene is available. Results of the quantitative PCR studies on the 266 samples are summarised in table 1.

Table 1. Correlation between quantitative PCR measurement of the mutant allele frequency and phenotype of the *P. viticola* samples

Range of the mutant allele percentage detected by quantitative PCR on P1 populations	Number of samples	Phenotype of the samples (R: QoI resistant, S: QoI sensitive)
$0 < x \leq 0.1$	78	S
$0.1 < x \leq 2$	57	S
$0.1 < x \leq 2$	11	R
$2 < x \leq 100$	120	R

In parallel to the first experiment on subcultured populations, the samples were analysed just after being harvested from the field, *i.e.* on P0 populations. At the P0 stage, the quantity of biological material is too limited to perform tests to identify the resistance phenotype. Moreover, the possible presence of chemicals on leaves might interfere with QoI resistant phenotype. In contrast, the quantitative PCR tool, is unaffected by these constraints. 187 of the 266 previous mentioned samples were also tested before any subculturing.
The correlation factor between the two quantitative PCR measurements is 0.81 indicating that there are similar mutant gene proportions both before and after subculturing. Results are shown graphically in figure 2.

The previously defined clustering (0-0.1, 0.1-2, >2 % mutant allele) is not that clearly respected. This is normal as the results are obtained from different sporangial populations. Six samples determined as QoI resistant with the quantitative PCR test on P0's, coming straight from the field become more or less sensitive after subculturing (P1) and eleven sensitive became resistant on subculture. In general, it seems that highly sensitive samples stay sensitive and that samples showing low levels of resistance become more resistant under laboratory subculturing conditions in the absence of any selection pressure. The differences observed in mutant allele quantification before and after subculture are probably due to sampling and particular fitness of the different samples although fitness does not link to QoI sensitivity.

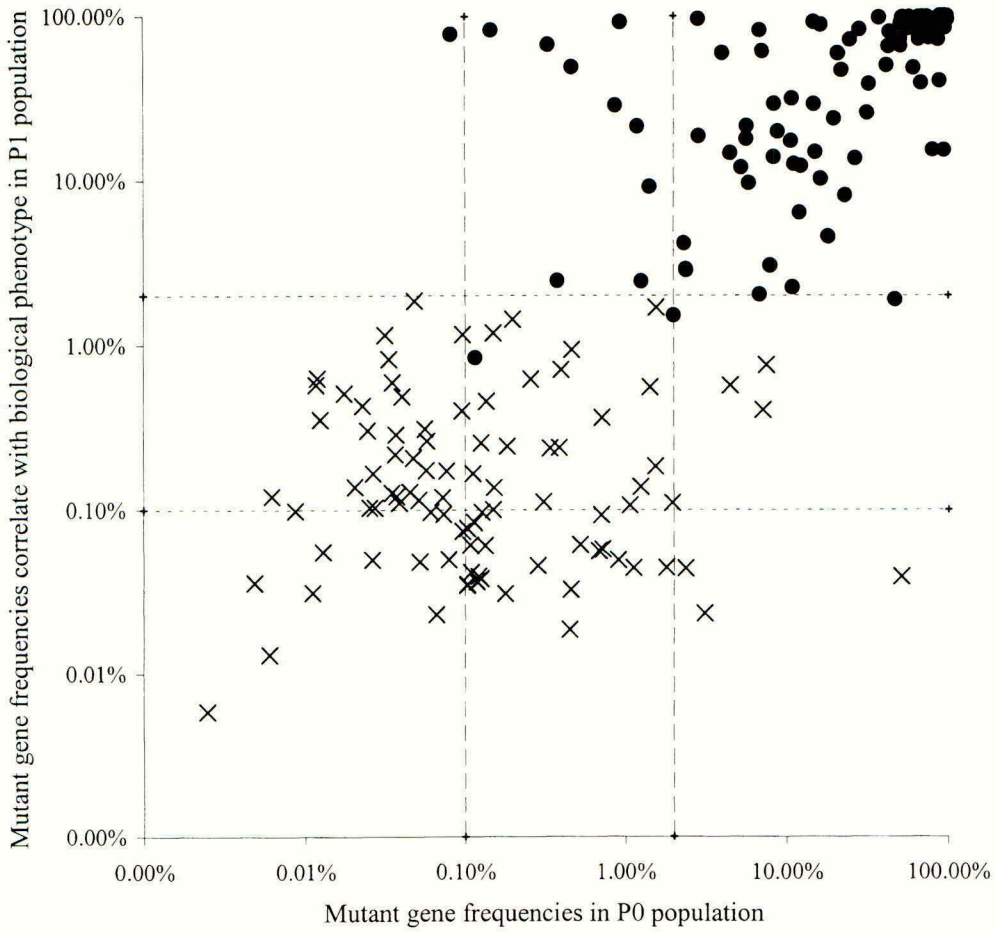

Figure 2. Correlation between mutant gene frequencies in samples before and after laboratory subculturing. Data is expressed on logarithmic scale to simplify presentation. Each strain is represented by a cross or a point where the phenotype is respectively QoI sensitive or resistant

DISCUSSION

A comparative analysis of the quantitative PCR measurement of mutant allele frequencies and the determination of QoI resistant phenotypes of several *P. viticola* samples has detected the presence of three classes. Samples sensitive to QoI fungicides containing less than 0.1% of mutant allele and resistant ones containing more than 2%. The samples in which mutant allele percentage is between these two limits are mostly sensitive as showed by biological determination. Therefore we have a majority of samples clearly identified as resistant or sensitive to QoI products thanks to the quantitative PCR test. Only 25% have to be tested in biology to confirm their phenotype. In conclusion, quantitative PCR is a reliable method to detect QoI resistance and has many advantages over classical biological techniques. Firstly, sample preparation and analysis is relatively fast: DNA extraction and mutation frequency quantification can be conducted in one day, compared to the 6 or 7 days required for growing strains with the biological test. Secondly, the quantity and the quality of the samples are not as important as in the biological test. Measurements are possible with very low quantity of samples, and are not affected even if samples are contaminated by normal chemical treatments in the fields.

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Interrelation between alternative respiration and target site mutations in resistance to QoI fungicides

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ABSTRACT

The two best understood mechanisms of fungal resistance to QoI fungicides are the induced expression of alternative respiration and mutational changes of the cytochrome *b* target site. The impact of both mechanisms on QoI sensitivities was investigated with *Magnaporthe grisea*. In the absence of a host, induced expression of alternative respiration provided effective rescue from azoxystrobin action for both conidia germination and mycelial growth. The disruption of a single alternative oxidase gene *AOXMg* yielded mutants fully deficient in the expression of alternative respiration. When surfaces of barley leaves were protected with azoxystrobin prior to the inoculation with conidia, symptoms were equally suppressed for both the wild-type strain and a *AOXMg*-minus mutant, indicating that alternative respiration was not induced on protected host surfaces. In contrast, the same mechanism allowed residual development of *M. grisea* when azoxystrobin was applied after infection. Residual development of mycelium provided by the induction of alternative respiration was also essential in the spontaneous emergence of highly resistant target site mutants. The results indicate that post-infection use of QoI fungicides might have fostered the emergence of such target site mutants.

INTRODUCTION

Commercial QoI fungicides disrupt mitochondrial electron transport by specifically binding to the Qo center of cytochrome *b* (Barlett *et al.*, 2002). Two potential mechanisms of QoI resistance have been studied in detail: rescue from inhibitor action *via* induction of alternative respiration and mutational changes of the cytochrome *b* target site (Barlett *et al.*, 2002; Heaney *et al.*, 2000; Köller *et al.*, 2001). Alternative respiration relies on the expression of alternative oxidase (AOX), an enzyme encoded by nuclear DNA and induced by reactive oxygen species accumulating when normal electron transport in mitochondria is blocked by a QoI (Joseph-Horne *et al.*, 2001; Köller *et al.*, 2001). Once AOX is incorporated into inner mitochondrial membranes, electrons will be transferred directly from

ubiquinol to oxygen, and the QoI target site will be circumvented. Thus far, this rescue mechanism has not been identified as the cause for the sometimes rapid selection of QoI-resistant pathogen populations. Instead, the G143A mutation of cytochrome *b* described first for a laboratory mutant of *Venturia inaequalis* (Köller *et al.*, 2001; Zheng *et al.*, 2000) was responsible (Bartlett *et al.*, 2002; Heaney *et al.*, 2000; Ishii *et al.*, 2001).

The disruption of a single alternative oxidase gene (*AOXMg*) of *Magnaporthe grisea* yielded mutants fully deficient in the alternative respiration response to QoI action (Avila-Adame & Köller, 2002). The study described below summarizes the impact of *AOXMg* disruption on both the *in vitro* and *in vivo* activities of the QoI azoxystrobin, and a potential interrelation between alternative respiration and the selection of QoI-resistant target site mutants.

MATERIALS AND METHODS

Strain 4091-5-8 of *M. grisea* pathogenic on goosegrass, weeping lovegrass and barley was used as a wild-type strain. The *AOXMg*-minus mutant M-145 was generated by gene disruption (Avila-Adame & Köller, 2002). Mutants of *M. grisea* resistant to azoxystrobin were generated by transferring fully melanized mycelium of 6-day-old wild-type cultures to agar medium amended with 10 μg ml^{-1} azoxystrobin plus 150 μg ml^{-1} salicylhydroxamic acid (SHAM) as an inhibitor of alternative respiration. A 628 bp cytochrome *b* fragment was prepared by PCR using 5'-GATCATATAGAGCTCCTCG-3' and '5-CTACCT-AAATCTGTAACAGG-3' as primers.

In vitro sensitivities of mycelia to azoxystrobin were determined as described (Avila-Adame & Köller, 2002). *In vitro* sensitivities of germinating conidia were determined with conidia suspensions retrieved from mycelial colonies. Suspensions were transferred to water agar containing azoxystrobin at concentrations ranging from 0.001 to 10 μg ml^{-1}. Germination was assessed after 24 hours. *In vivo* tests were performed with leaf segments of barley as described (Avila-Adame & Köller, 2002). In tests evaluating the protective activities of azoxystrobin, leaf segments were treated 24h prior to the inoculation with conidia. In post-infection tests, treatments were delayed until 36h after inoculation. The treatments reflected a pre-symptom mode of application, because first macroscopic symptoms appeared 48h after inoculation.

RESULTS

Responses of an *AOXMg*-minus mutant to azoxystrobin

As reported previously (Avila-Adame & Köller, 2002), mycelia of the wild-type strain of *M. grisea* employed in the study responded resistant to azoxystrobin by expressing alternative respiration. Disruption of *AOXMg* yielded mutants fully deficient in this rescue response. Based on ED_{50} values determined for the wild-type strain and the *AOXMg*-minus mutant M-145, alternative respiration provided a 180-fold rescue from azoxystrobin action

during the stage of mycelial growth (Table 1). An even more pronounced rescue response was measured for germinating conidia. Here, ED_{50} values separating the wild-type strain from mutant M-145 were different by a factor of >1,000 (Table 1).

Table 1. Sensitivity of *Magnaporthe grisea* to azoxystrobin under *in vitro* and *in vivo* test conditions. Sensitivities are expressed as ED_{50} values (µg ml^{-1})

	In vitro		***In vivo***	
Genotype	Mycelia	Conidia	Post-infection	Protective
Wild-type	1.8	>10	7.1	0.2
M-145	0.01	0.01	0.2	0.1
G143A	>10	>10	>10	>10
G143S	>10	>10	>10	>10

In vitro sensitivities of mycelia relate to post-infection activities of fungicides, while sensitivities of germinating conidia reflect a protective mode of fungicide application. The alternative respiration-deficient mutants of *M. grisea* allowed us to evaluate the impact of alternative respiration on azoystrobin activities in either a post-infection or a protective mode of azoxystrobin application. The post-infection ED_{50} value determined for the wild-type strain developing inside the host already was 36-times higher than for the *AOXMg*-minus mutant M-145 (Table 1). Although the rescue potential was less pronounced than for the *in vitro* growth of mycelia, the pertaining difference between the wild-type strain and the *AOXMg*-minus mutant indicated that alternative respiration was activated when azoxystrobin was applied to infected leaves. The data presented in Table 1 are based on the total of disease symptoms developing. It must be mentioned that the formation of fully mature lesions was suppressed more effectively (Avila-Adame & Köller, 2002).

In contrast to post-infection applications of azoxystrobin, the induction of alternative respiration was almost completely silenced when leaf surfaces were protected prior to the inoculation with conidia. Here, the rescue potential of >1,000 determined under *in vitro* conditions was reduced to a factor of 2 under *in vivo* conditions (Table 1). Complete control of symptom development was achieved at an azoxystrobin dose of 1 µg ml^{-1} for both the wild-type strain and the *AOXMg*-minus mutant

Spontaneous mutations toward azoxystrobin resistance

Mycelia expanding from the margins of *M. grisea* colonies are hyaline in appearance, while resting mycelia in the center of colonies are melanized. Sensitivities to azoxystrobin were

routinely measured by employing actively growing non-melanized mycelia retrieved from colony margins. Under such test conditions, an azoxystrobin dose of 10 µg ml^{-1} plus SHAM was fully toxic to mycelia of both the wild-type strain and the *AOXMg*-minus mutant. For melanized mycelial fragments, however, development was only prevented for the alternative respiration-deficient mutant, while residual although strongly inhibited colony growth was apparent for the wild-type strain (Fig. 1). After a second transfer of inhibited mycelia of the wild-type to media containing the same inhibitors, growth remained strongly suppressed for the majority of colonies. However, some of the colonies expanded rapidly (Fig. 1). Several of these spontaneous mutants were propagated as monoconidial isolates, and two of these monoconidial propagates expressed stable resistance to azoxystrobin plus SHAM. Such stable mutants appeared for 2% of colonies tranfered.

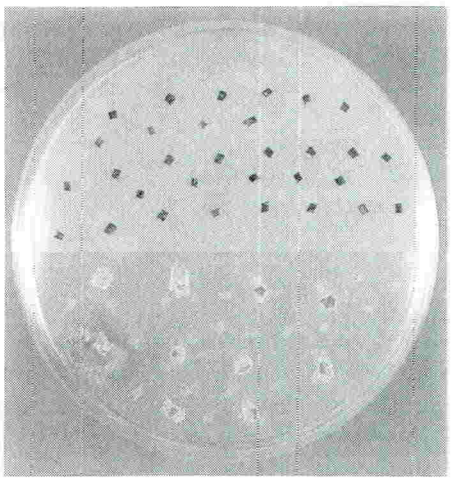

Figure 1. Origin of spontaneous mutants resistant to azoxystrobin. Melanized myceliua of mutant M-145 were fully inhibited after transfer to 10 µg ml^{-1} azoxystrobin plus SHAM (upper part). Residual growth giving rise to fast-growing mutants was observed for the wild-type strain under the same conditions (lower part).

Characterization of azoxystrobin-resistant mutants

The two stable mutants retrieved from rapidly expanding colonies of the wild-type strain (Fig. 1) expressed a high level of azoxystrobin resistance. Analysis of the cytochrome *b* gene revealed the presence of target site mutations. One mutant was distinguished by a GGT to GCT transversion giving rise to the G143A amino acid exchange affiliated with QoI resistance of several pathogens (Bartlett *et al.*, 2002; Heaney *et al.*, 2000). For the second mutant, the transversion was from GGT to AGT translating into a novel G143S mutation.

Mycelia and germinating conidia of both the G143A and G143S mutant resisted high doses of azoxystrobin under *in vitro* test conditions (Table 1). Inclusion of SHAM had no impact on this high level of resistance, indicating that expression of alternative respiration was not involved. Likewise, azoxystrobin failed to control disease symptoms in both post-infection and protective modes of application (Table 1).

DISCUSSION

For several pathogens, the selection of QoI-resistant subpopulations to levels of practical resistance progressed more rapidly than anticipated (Barlett *et al.*, 2002; Heaney *et al.*, 2000; Ishii *et al.*, 2001). In all cases, the G143A mutation in the cytochrome *b* target site documented for a laboratory mutant of *V. inaequalis* (Köller *et al.*, 2001) and here for *M. grisea* was affiliated with resistance expressed by field isolates. Thus far, the rescue mechanism inherent to alternative respiration has not been found to be involved in the expression of QoI resistance. The low practical impact of alternative respiration has been explained by a model implying that plant antioxidants such as flavone will quench reactive oxygen species required for the induction of alternative oxidase (Mizutani *et al.*, 1996). Consequently, alternative respiration would be silenced when a pathogen develops inside a host plant.

Our approach to directly assess this model was to disrupt a single alternative oxidase gene in *M. grisea*, thereby generating alternative respiration-deficient but otherwise isogenic and fully pathogenic mutants (Avila-Adame & Köller, 2002). For *M. grisea*, the QoI rescue mechanism inherent to the induction of alternative respiration was most effective during the stage of conidia germinating in the absence of a host. Surprisingly, the rescue mechanism was almost completely silenced when conidia infected a host surface after it was protected with azoxystrobin. In contrast, induction of alternative respiration continued to rescue mycelia already established inside the host before they were challenged with azoxystrobin. Mechanisms involved in this differential silencing of alternative respiration under different *in vivo* conditions remain to be determined.

Residual growth of mycelium in the presence of high azoxystrobin doses was also essential in the spontaneous and frequent appearance of highly resistant target site mutants. The generation of such mutants could be explained by the replacement of preexisting mutated alleles of the mitochondrial cytochrome *b* gene, or to mutations mediated during prolonged yet non-lethal exposure to the QoI. Both the G143A and the G143S mutations were based on transversions of guanine, a nucleotide known to be oxidized by reactive oxygen species and, in its oxidized form, responsible for mutations in mitochondrial DNA (Sawyer & van Houten, 1999). Reactive oxygen species acculmulate in mitochondria when QoIs are present (Joseph-Horne *et al.*, 2001; Köller *et al.*, 2001).

Regardless of the precise mechanism involved in the spontaneous emergence of the target site mutants described here, they required residual growth of QoI-treated cells provided by alternative respiration. Such residual growth was also provided in post-infection but not in

protective applications of azoxystrobin to barley leaves challenged by *M. grisea*. Post-infection applications of QoI fungicides might, therefore, have contributed to the selection and perhaps even the generation of stable target site mutants. Consequently, future anti-resistance strategies should enforce the restriction of QoI use to protective modes of applications.

ACKNOWLEDGEMENTS

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Sensitivity of European isolates of *Phytophthora infestans* to famoxadone and cymoxanil

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ABSTRACT

In 2001, sixty *P. infestans* isolates originating from Poland, UK and Germany were evaluated *in vitro* for their sensitivity to the fungicides famoxadone and cymoxanil. Sensitivities were determined by measuring radial growth on agar medium amended with fungicide. All isolates were fully sensitive to famoxadone compared to pre-commercialization baseline isolates. The same level of sensitivity was recorded in Poland, Germany and the UK. *P. infestans* isolates originating from famoxadone-treated fields were as sensitive as those from untreated plots indicating no seasonal shift in sensitivity. No decrease in sensitivity to cymoxanil was recorded in 2001 compared to previous years despite frequent use of cymoxanil-based mixtures in these countries. Within-field variation was low and in the same order of magnitude as that found for isolates originating from different potato fields within the same country.

INTRODUCTION

Fungicide resistance is a serious problem in disease management, particularly in the case of potato late blight caused by *Phytophthora infestans*. Resistance to phenylamides such as metalaxyl developed soon after their introduction and still occurs today in many potato areas. In order to maintain the effectiveness of fungicides over many years, good resistance management practices must be observed. For example, late blight fungicides are generally used in mixtures and/or alternation with other fungicides with different modes of action.

Famoxadone (Famoxate, DPX-JE874) is a new fungicide recently introduced in several European countries for the control potato and tomato late blight (Joshi & Sternberg, 1996). The fungicidal activity of famoxadone is through inhibition of the mitochondrial cytochrome bc1 complex at the Qo center and the fungicide is currently referred to as a Qo inhibitor (QoI) (Jordan, *et al.*, 1999). Although initial assessment studies indicated that the risk of development of resistance to Qo inhibitors was moderate or low, cases of QoI resistance have already been reported in several populations of plant pathogens (Heaney, *et al.*, 2000). For late blight control, famoxadone is only available in mixture with other fungicides such as cymoxanil (Curzate), an active ingredient with a different mode of action and used for more than 20 years against this disease. Monitoring work conducted in the 1990's demonstrated no decrease in sensitivity to cymoxanil among global populations of *P. infestans* (Hamlen & Power, 1998).

The objective of this study was to investigate the level of sensitivity of European *P. infestans* populations in areas where famoxadone has been introduced. At the same time, sensitivity to cymoxanil was assessed as part of a routine resistance monitoring program.

MATERIALS AND METHODS

P. infestans isolates were collected in 2001 from potato fields in Germany, UK and Poland. A total of 60 single-lesion isolates were obtained and immediately tested for sensitivity to famoxadone and cymoxanil in a radial growth assay on rye B agar (Power, *et al.*, 1995). The fungicides were dissolved in DMSO before incorporation into the medium at final concentrations ranging from 0.05 to 2 mg/litre. Mycelial plugs on agar were obtained from the actively expanding margin of a 7-day old culture and transferred to the test media. Five replicates were made for each fungicide concentration. After 10 days of incubation at 19°C in the dark, the diameter of the mycelial colony was measured. The dose-response data was subjected to probit analysis and EC_{50} values calculated for each fungicide and isolate. Assay consistency was validated by using two reference *P. infestans* isolates with different levels of sensitivity to famoxadone.

In order to detect seasonal shifts in sensitivity, isolates were sampled from potato fields that had been either treated or not treated with famoxadone in 2001. Within-field variation was assessed by determining sensitivity levels to both fungicides in one location in Germany (11 isolates originating from the same field) and one location in Poland (10 isolates).

RESULTS

Sensitivity to famoxadone

For famoxadone, the EC_{50} values of the 60 *P. infestans* isolates ranged from 0.035 to 0.552 mg/litre with an average of 0.179 mg/litre (Table 1).

Table 1. Distribution of sensitivities to famoxadone of *P. infestans* isolated from potato fields in 2001

Country	No. of isolates	EC_{50} (mg/litre)		Distribution Factor
		Mean± STD	Range	
Germany	42	0.191±0.13	0.045-0.487	10.8
UK	8	0.152±0.11	0.053-0.552	10.4
Poland	10	0.136±0.08	0.035-0.316	9.0
Total	60	0.179±0.12	0.035-0.552	15.8

None of the isolates exhibited EC_{50} values greater than that of the isolate used as a reference in these tests (1.07 mg/litre). Mean EC_{50} values were comparable in Germany, UK and Poland with similar distribution factors (DF = maximum EC_{50} value/minimum EC_{50} value) in the order of 10-fold for all 3 countries. The sensitivity profile to famoxadone was the same (P = 0.05) whether the isolates originated from fields treated (29 isolates) or not treated (31 isolates) with the mixture famoxadone + cymoxanil in 2001 (Figure 1).

The width of the sensitivity distribution for the 2001 *P. infestans* isolates was narrower than that of the baseline isolates sampled between 1990 and 1995 i.e. prior to the introduction of famoxadone in these countries (Figure 2). Also, the mean EC_{50} value for the 2001 isolates (0.179 mg/litre) was somewhat lower than that of baseline isolates (0.40 mg/litre).

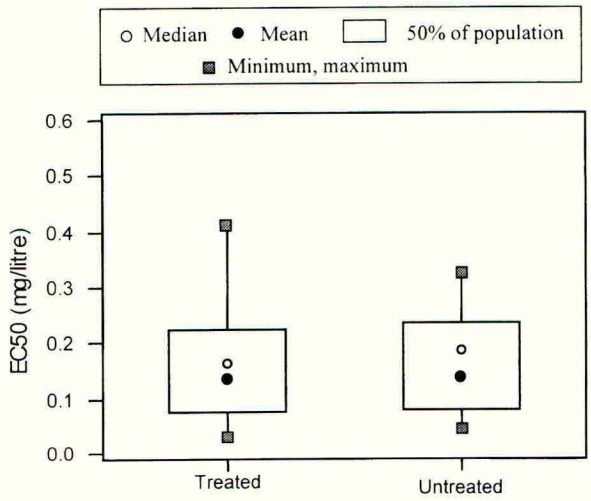

Figure 1. Sensitivity profile for famoxadone of *P. infestans* isolates originating from potato fields untreated or treated with the mixture famoxadone + cymoxanil

Sensitivity to cymoxanil

The reaction of the 60 *P. infestans* isolates to cymoxanil was similar to that found for famoxadone using the same assay methodology. The mean EC_{50} values for cymoxanil (0.087 mg/litre) was approximately half that found for famoxadone with little variation between the countries investigated (Table 2). The distribution factor (DF=17.5) was nearly the same as that for famoxadone.

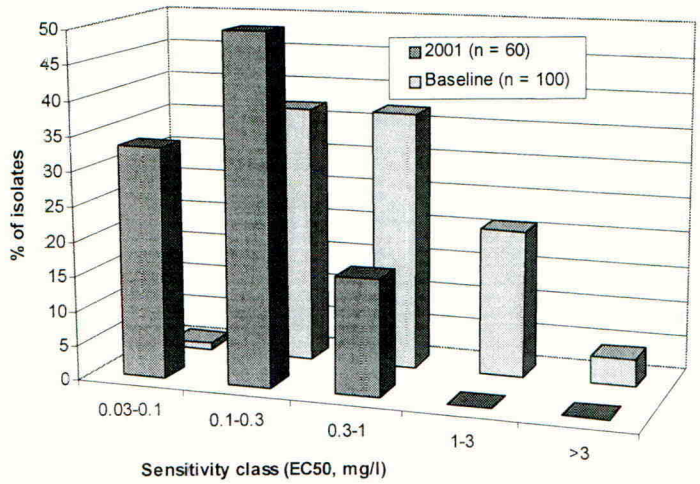

Figure 2. Distribution of sensitivities to famoxadone for 2001 European *P. infestans* isolates (n = 60) compared to baseline isolates (n = 100)

Within-field variation

There was some variation in the level of sensitivity of *P. infestans* isolates originating from the same potato field. The distribution factors for famoxadone and cymoxanil was in the same order of magnitude for isolates obtained in one location in Germany and in Poland (Table 3).

Table 2. Distribution of *in vitro* sensitivity to cymoxanil of *P. infestans* strains isolated from potato fields in Germany, UK and Poland in 2001.

Country	No. of isolates	EC_{50} (mg/litre)		Distribution Factor
		Mean± STD	Range	
Germany	42	0.087±0.05	0.013-0.228	17.5
UK	8	0.090±0.05	0.054-0.209	3.9
Poland	10	0.086±0.05	0.029-0.175	6.0
Total	60	0.087±0.05	0.013-0.228	17.5

Table 3. Within-field variability of *P. infestans* sensitivities to famoxadone and cymoxanil in two individual potato fields

Site	No. of isolates	EC_{50} for famoxadone (mg/litre)		EC_{50} for cymoxanil (mg/litre)	
		Mean±STD	Distribution factor	Mean±STD	Distribution factor
Biblis (Germany)	11	0.119±0.07	5.2	0.087±0.07	10.8
Bonin (Poland)	10	0.141±0.08	7.2	0.061±0.03	4.2

DISCUSSION

The data for the 60 *P. infestans* isolates collected in 2001 indicate no decrease in sensitivity to famoxadone compared to baseline isolates from 1990-1995 (Figure 2). The distribution of EC_{50} values for the 2001 isolates (DF=17.5) was in fact narrower than that observed for European baseline isolates (DF=119) (Hamlen, *et al.*, 1998). In comparison, significantly greater distribution factors have been reported for isolates collected in Mexico (DF=1000) which is consistent with the genetic diversity reported in Mexican *P. infestans* populations (Goodwin, *et al.*, 1992). Sensitivity of the 2001 *P. infestans* isolates was not significantly different between the three countries investigated despite the fact that famoxadone has been used commercially for three years in Poland compared to only one season of use in UK and Germany. Isolates originating from famoxadone-treated field (up to 6 applications) had similar sensitivity profiles compared to isolates from untreated fields indicating no selection of less sensitive *P. infestans* isolates following exposure to famoxadone in these fields. These results conform to expectations from risk assessment studies conducted in France and Mexico where repeated use of famoxadone alone (up to 18 applications per season) did not result in detectable shifts in sensitivity (Hamlen, *et al.*, 1998). Within-field variation was in the same order of magnitude as that found for isolates originating from different potato fields within the same country. This level of variation was significantly greater than assay-to-assay variability (data not shown) and could reflect the genetic diversity within a sexually reproducing *P. infestans* population in a potato field.

Cases of QoI resistance have been reported for several plant pathogenic fungi such as cereal powdery mildew (*Blumeria graminis*), apple scab (*Venturia inaequalis*) and downy mildew of cucurbits (*Pseudoperonospora cubensis*) (Heaney, *et al.*, 2000). Several cases of resistance to Qo inhibitors have also been reported among European populations of grape downy mildew (*Plasmopara viticola*), only a few years following commercial introduction of these fungicides (FRAC: www.gcpf.org/frac/QoIWG.html). Qo fungicides including straight strobilurins have been used more intensively on these crops. Famoxadone and fenamidone, a related compound,

are the only Qo inhibitors currently registered in Europe for the control of potato late blight and their use has been relatively limited so far. According to current recommendations, QoI-based mixtures should not be applied more than 6 times per season on a potato crop or no more than 50% of the total number of sprays, whichever is the lower. Furthermore, the number of consecutive applications is limited to three (FRAC webpage). It is important that growers and advisors observe these recommendations strictly in order to delay the development of resistance to these fungicides.

Famoxadone is made available in pre-mixtures with cymoxanil, another fungicide effective against *P. infestans*. Cymoxanil is not related either chemically or by cross-resistance to famoxadone (Joshi & Sternberg, 1996) and has a long history of use for potato and tomato late blight control. The EC_{50} values of the 60 *P. infestans* isolates analysed in this study were well within the range of sensitivities reported for baseline isolates obtained from laboratory collections and tested in the radial growth assay (Hamlen & Power, 1998). Sensitivity data from a leaf disc assay revealed a significantly larger variation in sensitivity to cymoxanil (DF=1000) compared to that reported here. No resistant isolates were found, however, using that method (Gisi, *et al.*, 1997). Our data supports the conclusion that no resistance to cymoxanil is occurring among *P. infestans* populations in the three countries investigated.

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Shift in sensitivity of *Alternaria solani* (potato early blight) to strobilurin fungicides

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ABSTRACT

Forty-seven *Alternaria solani* isolates collected from 1998 and 2001 from various potato growing regions in the United States were assayed *in vitro* for sensitivity to azoxystrobin. Twenty-one *A. solani* isolates collected in 1998, prior to the introduction of azoxystrobin, had a mean baseline EC_{50} value of 0.0279µg/ml. Isolates of *A. solani* collected in 2001, recovered from fields displaying a lack of disease control by azoxystrobin, had a mean EC_{50} of 0.3480µg/ml. Mean EC_{50} values for baseline isolates to pyraclostrobin and trifloxystrobin were 0.0022µg/ml and 0.0060 µg/ml respectively. In 2001, sensitivities to pyraclostrobin and trifloxystrobin shifted to mean EC_{50} values of 0.0208µg/ml, and 0.0140µg/ml respectively. *In vivo* assessments of sensitivity to azoxystrobin and pyraclostrobin were conducted on six isolates selected from the *in vitro* cross-resistance evaluations. Results from the *in vivo* assays were correlated to those obtained in the *in vitro* assays. Field studies need to be conducted to determine if the shift in sensitivity to pyraclostrobin and trifloxystrobin will result in a similar loss of disease control under commercial potato growing conditions as observed with azoxystrobin.

INTRODUCTION

Late in the 1998 growing season, Minnesota, North Dakota, Nebraska, and Wisconsin were granted emergency use registration for azoxystrobin on potato (Quadris, Syngenta Crop Protection, Greensboro, NC), full registration was granted in 1999. Initially, excellent early blight control was provided by this new chemistry, however reduced disease control was observed in isolated commercial potato fields in 2000. Strobilurins have a single site mode of action, targeting electron transport of the cytochrome bc_1 complex. Related strobilurin chemistries, trifloxystrobin (Gem, Bayer), registered for use on potato, and pyraclostrobin (Headline, BASF, registration pending), have the same mode of action.

The objectives of this study include; (a) establishment of the baseline sensitivity of *A. solani* to azoxystrobin; (b) examination of *A. solani* isolates collected in 2001 to determine if a shift in sensitivity to azoxystrobin is responsible for the lack of disease control in the field; (c) evaluation of *A. solani* sensitivity to pyraclostrobin, and trifloxystrobin; and (d) determination of the biological significance of the *in vitro* shift in *A. solani* towards strobilurin insensitivity.

MATERIALS AND METHODS

Isolate Collection

Isolates of *A. solani* were recovered via single spore isolation from naturally infected potato foliage and tuber tissue submitted from various potato growing regions throughout the United States in 1998 and 2001.

In vitro Fungicide Sensitivity

The 50% effective concentration (EC_{50}) of each isolate was determined by evaluating spore germination on water agar amended with technical grade fungicide (0, 0.001, 0.01, 0.1, and 1.0µg/ml) and 100µg/ml salicylhydroxamic acid (SHAM). Conidia were dislodged from 12 to 14 day-old cultures grown on Clarified V-8 (CV-8) medium. The concentration of the conidial solution was adjusted to $2x10^5$ conidia/ ml, applied to the agar plates and incubated for 4 hours under continuous light at 20C. A conidium was considered germinated if the germ tube was at least equal in length to the conidium or if there were multiple normally developing germ tubes. Each fungicide assay was performed at least twice for each *A. solani* isolate.

In vivo Fungicide Sensitivity

Six isolates of *A. solani* previously tested *in vitro* for sensitivity to azoxystrobin and pyraclostrobin, were evaluated *in vivo*. Tomato plants, cv. Bonny Best, were inoculated with approximately 1ml of a $2.0x10^5$ spore/ml spore suspension when the first true leaves were well developed. Plants were treated with commercial formulations of azoxystrobin and pyraclostrobin at concentrations of 0, 0.1, 1.0, 10.0, and 100.0µg/ml. Twenty-four hours after fungicide application, tomato plants were inoculated and placed into mist chambers (>95% RH, 22-24C, 16 hour photoperiod). After 24 hours plants were returned to the greenhouse. Disease severity was determined 14 days post-inoculation. *In vivo* sensitivity assays were performed three times.

RESULTS

In vitro Fungicide Sensitivity

Emergency use labels for azoxystrobin were granted and first applications were made after 30 July 1998 in MN, ND, NE, and WI, and full label registration was not available in other states until 1999, therefore, only isolates collected prior to these dates in the respective states were included in the baseline assessment. Baseline EC_{50} values for azoxystrobin ranged from 0.0185 to 0.0340µg/ml, with a mean EC_{50} of 0.0279µg/ml among the twenty-one isolates collected in 1998 (Figure 1). Among the twenty-six isolates assayed from 2001, twenty-three of the isolates were recovered from fields displaying a lack disease control by azoxystrobin. The EC_{50} values among these isolates ranged from 0.1450µg/ml to 0.6250µg/ml, with a mean of 0.348µg/ml, representing more than ten-fold shift in sensitivity to azoxystrobin. The remaining three isolates from 2001 were collected from a field in which azoxystrobin was effectively controlling early blight disease. The EC_{50} values of these three isolates were 0.0125µg/ml, 0.0305µg/ml, and 0.0340µg/ml respectively, below or within the range of

baseline EC_{50} values. The difference in sensitivity to azoxystrobin between the 1998 and 2001 populations was significant (P=0.001).

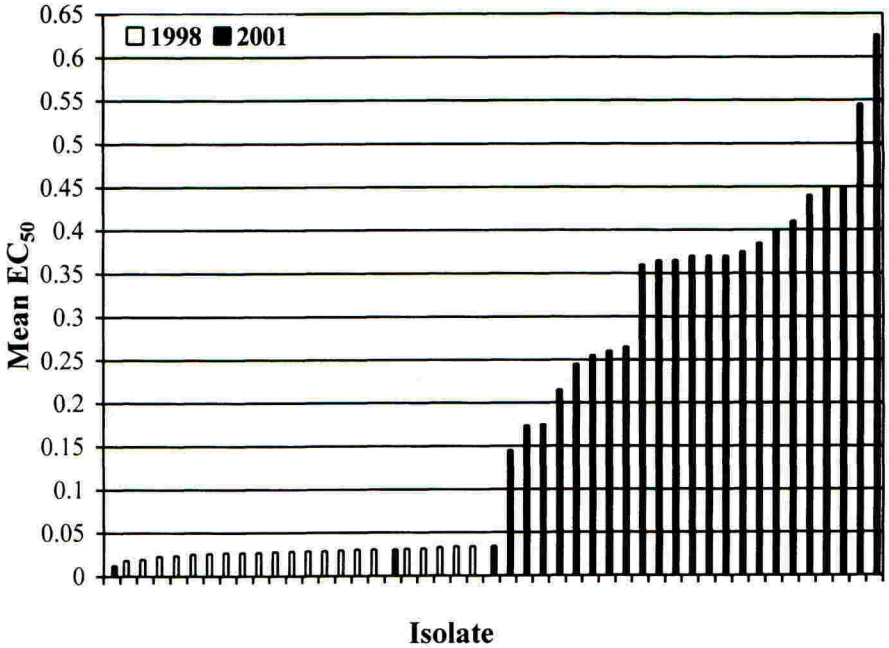

Figure 1. In vitro sensitivity of 1998 baseline isolates and isolates collected in 2001 of *Alternaria solani* to azoxystrobin (spore germination assay). Isolates are ranked in order by sensitivity to azoxystrobin.

A. solani isolates were further examined *in vitro* for sensitivity to pyraclostrobin and trifloxystrobin. The mean EC_{50} value of baseline isolates for pyraclostrobin was 0.0022µg/ml, while the mean for the 2001 isolates was 0.0208µg/ml, representing nearly a ten-fold shift in sensitivity (Figure 2). The mean EC_{50} for baseline isolates to trifloxystrobin was 0.0060µg/ml, and the mean for the 2001 isolates was 0.0140µg/ml, only a two-fold shift in sensitivity (Figure 3). Again, the difference between 1998 and 2001 isolates was significant (P=0.001) for both pyraclostrobin and trifloxystrobin.

In vivo Fungicide Sensitivity

In vivo assessments of *A. solani* sensitivity to these three fungicides were conducted on six isolates selected from the *in vitro* cross-resistance evaluations. Dose response curves indicate that for the baseline population, the sensitive isolates of *A. solani*, azoxystrobin and pyraclostrobin provide very similar disease control. However, reduced sensitive populations were controlled more effectively with higher fungicide concentrations of pyraclostrobin compared to azoxystrobin (Figure 4). *In vitro* and *in vivo* assays were correlated for azoxystrobin (r=0.901, P=0.014), and pyraclostrobin (r=0.842, P=0.035).

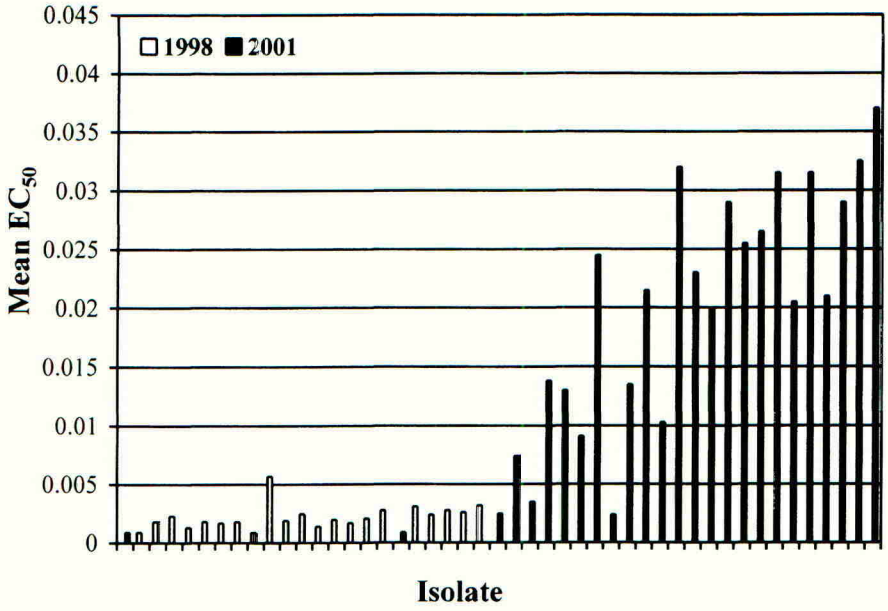

Figure 2. In vitro sensitivity of 1998 baseline isolates and isolates collected in 2001 of *Alternaria solani* to pyraclostrobin (spore germination assay). Isolates are ranked in order by sensitivity to azoxystrobin.

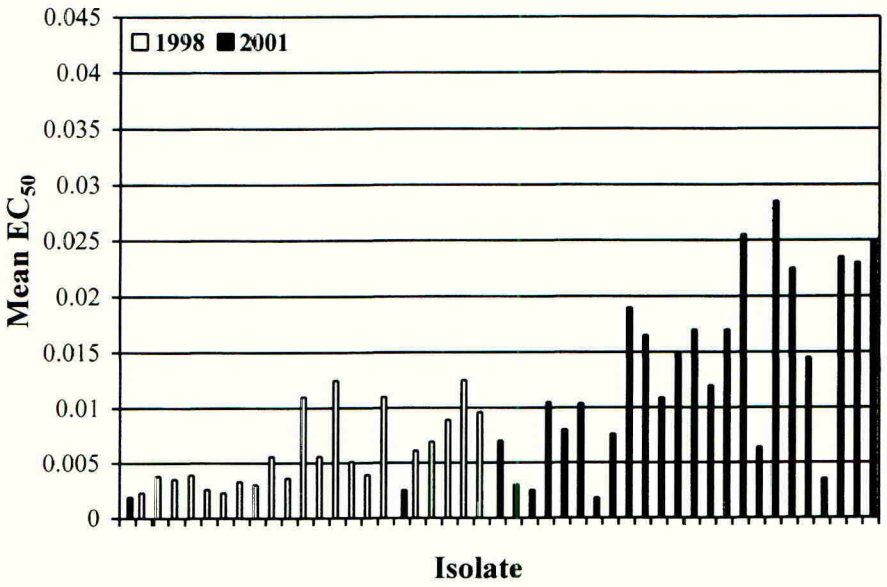

Figure 3. In vitro sensitivity of 1998 baseline isolates and isolates collected in 2001 of *Alternaria solani* to trifloxystrobin (spore germination assay). Isolates are ranked in order by sensitivity to azoxystrobin.

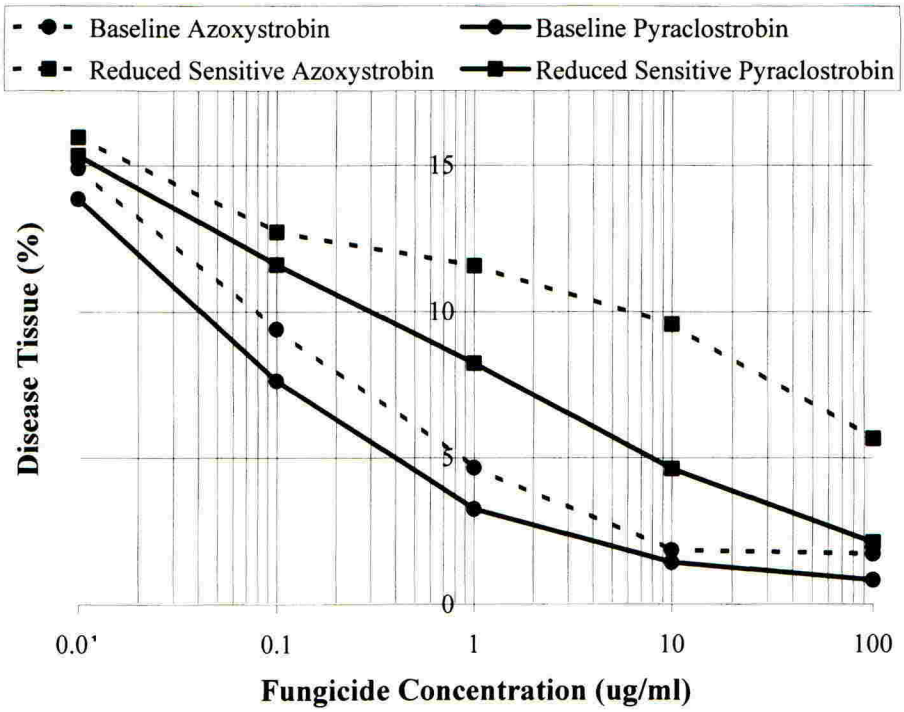

Figure 4. Mean *in vivo* dose response curves for three baseline, and three reduced sensitive isolates of *Alternaria solani*.

DISCUSSION

A shift in *in vitro* and *in vivo* sensitivity was detected for azoxystrobin, confirming that the lack of disease control observed in commercial fields in 2000 and 2001 was due to decreased efficacy of the fungicide where reduced sensitive populations of *A. solani* were present. This lack of control was first observed in Nebraska in a potato-growing region where conditions are ideal for the development of early blight. The cultivar, Russet Norkotah, is extremely susceptible to early blight, and weather conditions provide alternating wet and dry periods, sometimes two in one day, through the development of dew, and frequent overhead irigations. *A. solani* on potato requires interrupted wetting periods to efficiently sporulate. This cycle of induction occurs between the first wetting period when conidiophores are formed and the second wetting period which is required for spore production (Rotem, 1994). Because of these conditions, many growers in this region have applied the maximum registered amounts of azoxystrobin since the emergency use label was granted in 1998.

The shift in *in vitro* sensitivity of *A. solani* to all three strobilurin fungicides included in this study was significant, but this shift was much more evident with both azoxystrobin and pyraclostrobin and elevated across all *A. solani* isolates than what was observed with trifloxystrobin. Sensitivity of *A. solani* to pyraclostrobin and azoxystrobin was also highly

correlated *in vivo*. It remains to be seen if the shift in sensitivity to pyraclostrobin, which was not as pronounced as azoxystrobin at higher fungicide concentrations in the *in vivo* assays, will result in the same loss of disease control in the field.

Resistance to strobilurin fungicides has been reported in a number of plant pathogens. The cytochrome b gene has been cloned and sequenced for several of these plant pathogenic fungi including; *Venturia inaequalis* (Zheng and Köller 1997), *Erysiphe graminis* f. sp. *tritici* (Sierotzki *et al.* 2000b), *Pyricularia grisea* (Farman 2001), *Pseudoperonospora cubensis* (Ishii *et al.* 2001), *Podosphaera fusca* (Ishii *et al.* 2001), and *Mycosphaerella fijiensis* (Sierotzki *et al.* 2000a). When the DNA sequence of the cytochrome b gene was compared in sensitive and strobilurin resistant field isolates of *E. graminis*, *P. cubensis, P. fusca, and M. fijiensis,* only one single-point mutation leading to an amino acid change at position 143 from glycine (G) to alanine (A) was identified. Six strobilurin resistant isolates of *P. grisea* possessed the G143A mutation, while two isolates, which were partially controlled *in vivo* by strobilurins, possessed a mutation at position 129 from phelylalanine (F) to leucine (L) (Vincelli 2002). Six isolates from our 2001 *A. solani* collection have been sequenced and all possessed the F129L mutation while the baseline isolate did not (G. Olaya, Syngenta Crop Protection, personal communication). None of the isolates possessed the G143A mutation. As with *P. grisea*, these isolates are partially controlled *in vivo* by strobilurins.

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Mefenoxam resistance in the North American population of *Phytophthora erythroseptica*: Spatial distribution and frequency of resistance in soil and recombinant populations

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ABSTRACT

Mefenoxam is currently the only fungicide used to control pink rot, an important soil-borne disease of potato caused by *Phytophthora erythroseptica*. Isolates obtained from infected tubers collected throughout North America range widely in their responses to mefenoxam. Of 1168 isolates tested, 75.9% were classified as "sensitive" (S) – (EC_{50} <1.0 μg ml^{-1}), 3.9% as "intermediate" (I) – (EC_{50} 1.0 – 99.9 μg ml^{-1}) and 20.2% as "resistant" (R) – (EC_{50} ≥100 μg ml^{-1}). Further studies were initiated to examine the frequency of resistance as it exists in soil populations of the pathogen and to monitor its changes in frequency distribution over time. In 1998, 87.9% of the 29 isolates collected from 6 sites within a field known to be infested with *P. erythroseptica* were found to be mefenoxam-resistant. In 2000, isolates were recovered from 4 of the 6 sites. All isolates recovered from sites yielding R isolates in 1998 were resistant in 2000. The population at one site shifted from 92.3% S and 7.7% I types in 1998 to 0.0% S, 5.0% I, 95.0% R in 2000. "Intermediate" isolates represent a small proportion of the population. Populations developed from selfed isolates (S, I, and R) were non-segregating. The EC_{50}s of F1s obtained from S parental isolates all fell into the "sensitive" range with a slight shift toward increased sensitivity while the progeny of the R isolates were found to be "resistant", ranging in a normal distribution around the EC_{50}s of the respective parental isolates. Greater variability was observed in the F1 populations obtained from I parental types with a shift toward resistance occurring in the populations collected from the isolates with the higher EC_{50} values.

INTRODUCTION

Pink rot, caused by *Phytophthora erythroseptica*, is an important soil-borne disease of potato. The disease can be found during or immediately following harvest, especially in areas and in years with high moisture and can be widely distributed in potato fields and storage bins. Strategies used to control pink rot often involve rigorous management of cultural practices such as crop rotation, planting the potato crop in well-drained soils, avoiding excessive irrigation at the end of the growing season and modifying handling procedures to reduce wounding. Mefenoxam (Ridomil Gold® EC and Ultrafluorish® EC) is the only fungicide currently available that will effectively control the disease but its effectiveness may be compromised if insensitive isolates of *P. erythroseptica* become prevalent.

Metalaxyl-resistant isolates have been discovered in Maine, New York, Idaho and Minnesota in the United States during surveys of infected tubers (Goodwin and McGrath, 1995; Gudmestad *et al.*, 2000; Lambert and Salas, 1994; Taylor *et al.*, 2002a,b). Isolates of *P. erythroseptica* also have been collected from soil and the distribution of mefenoxam resistance

in these soil populations is consistent with that found during the tuber survey (Taylor *et al.*, 2002b). In highly infested fields, the pathogen can be distributed uniformly throughout while concentrating in some very small, localized "hot spots". Further studies were initiated to assess temporal distribution of mefenoxam sensitivity in soil populations of *P. erythroseptica*. Several isolates showing "intermediate" sensitivity to mefenoxam have been collected in these studies. Additionally, genetic diversity was examined by determining the range of sensitivity that occurs in F1 populations of the pathogen.

MATERIALS AND METHODS

Fungicide sensitivity screening

Isolates were screened on modified V8 medium (5% V8 juice filtered through four layers of cheesecloth and 2% agar) amended with metalaxyl in 1997 and mefenoxam in 1998-2001 according to the procedure described previously (Taylor *et al.*, 2002a). The relative growth reduction for each rate of fungicide was calculated as follows: [100-(growth with fungicide/growth in control plate)*100]. The concentration causing 50% relative reduction of mycelial growth (EC_{50}) compared to the control was estimated by plotting the percent inhibition against the log-scale of fungicide concentration or by regression curve fitting.

Isolation of *P. erythroseptica* from soil

In 1998, soil samples were taken from a field known to be infested with R isolates and their locations were recorded via a portable 12 channel Global Positioning System (GPS) receiver. Soil was collected from within the planted row (C) and in the empty furrows adjacent laterally to the row (E, W) at each of these sites again in 2000. Potato tubers (*cv.* Russet Norkotah) were used as trap host material to isolate the fungus from the soil samples. Cylinders (40 mm by 7 mm) were excised from whole tubers using a #3 cork borer. The space created by removing the tissue was filled with a subsample (approximately 2.0 g of soil) taken from a field sample. The soil was lightly packed then moistened to saturation with 6 - 8 drops of soil extract. Soil extract was prepared by autoclaving an aqueous slurry of field soil (100 g litre^{-1} of deionized H_20). The moistened subsample was capped with a disk (2 mm thick) cut from the end of the tissue cylinder (including periderm). After incubation in covered, moist chambers boxes (10 days at 20°C), tubers were removed and cut, bisecting the soil subsample through the long axis. Tubers exhibiting pink rot symptoms were counted and set aside. The pathogen was isolated from infected tubers and tested for mefenoxam sensitivity as described above.

P. erythroseptica – F1 populations

Sensitive, intermediate and resistant isolates were selected as parents to produce F1 generations. Each parental isolate was selfed and 100 single oospore cultures representing F1 progeny were established using a procedure similar to that of Medina and Platt (1999). Oospore suspensions (6-7 drops) were pipetted to water agar plates containing ampicillin (200 µg ml^{-1}). The plates were sealed with parafilm and incubated (20-25°C) under florescent lights for 2 days. Single oospore cultures were established by transferring individual germinated oospores to V8 juice agar medium (10% V8 juice filtered through four layers of cheesecloth, 0.1% $CaCO_3$ and 1.5% agar) containing ampicillin (200 µg ml^{-1}). Mefenoxam sensitivity of each F1 isolate was evaluated to determine the phenotypic make up of that generation.

RESULTS AND DISCUSSION

A total of 805 isolates of *P. erythroseptica*, collected during a 4-year, multi-location survey of infected tubers, were screened for mefenoxam sensitivity (Taylor *et al.*, 2002a,b). Figure 1 summarizes the results of that survey and includes data from an additional 5th survey year. The previously untested isolates increased the total number screened to 1168 and of these, 75.9% were classified as "sensitive" (S) – (EC_{50} <1.0 μg ml^{-1}), 3.9% as "intermediate" (I) – (EC_{50} 1.0 – 99.9 μg ml^{-1}) and 20.2% as "resistant" (R) – (EC_{50} ≥100 μg ml^{-1}). Resistant isolates (42) comprised 67.7% of the population from Maine. The 161 R isolates from Idaho represented 39.7% of the population from that state while a much smaller proportion (6.8%) from Minnesota (33) was confirmed to be mefenoxam resistant. Isolates classified as "intermediate" were identified but these were only found in the three states where R isolates were present. Isolates sensitive to mefenoxam were present in all twelve survey areas.

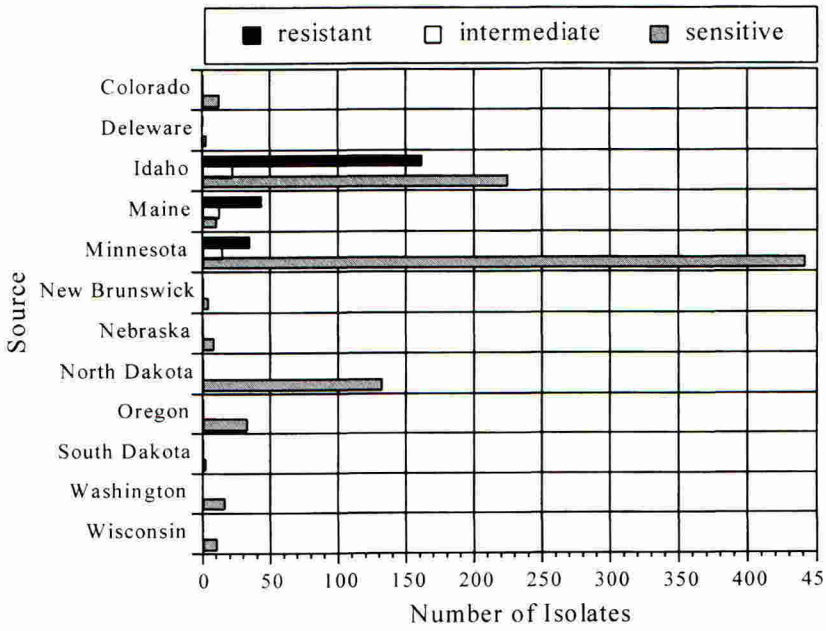

Figure 1. Mefenoxam sensitivity of *P. erythroseptica* isolates collected during a survey (1997-2001) of infected tubers obtained from the United States and Canada.

In 1998, 29 isolates were collected from 6 locations within a field known to be infested with *P. erythroseptica* and a large proportion of the soil population (87.9%) was found to be mefenoxam-resistant (Table 1). These R isolates comprised 100% of the population at 5 of the sampling sites. Nearly all (11 of 12) of the isolates from Site 8 were found to be sensitive to mefenoxam. A single I isolate was collected that year (also from Site 8). In 2000, isolates were recovered from 4 of 6 sites. All isolates from sites yielding R isolates in 1998 were resistant in 2000. The Site 8 population shifted from 92.3% S, 7.7% I, 0.0% R types (1998) to 0.0% S, 5.0% I, 95.0% R two years later. No differences in the frequency of resistance were observed in the isolates recovered from lateral furrows vs. mefenexom-treated planted rows.

Table 1. Mefenoxam sensitivity of *P. erythroseptica* isolates obtained from soil.

Year	Site #	Location	Isolates	Mefenoxam Sensitivity of Isolates (EC_{50} - μg ml^{-1})		
				< 0.05 – 0.99	*1.0 - 99.9*	*>100*
				sensitive	*intermediate*	*resistant*
1998	7	-	5	0	0	5
2000	7	E	3	0	0	3
	7	C	12	0	0	12
	7	W	4	0	0	4
1998	8	-	13	12	1	0
2000	8	E	7	0	1	6
	8	C	1	0	0	1
	8	W	12	0	0	12
1998	9	-	1	0	0	1
2000	9	E, C, W	0	nr	nr	nr
1998	13	-	5	0	0	5
2000	13	E	2	0	0	2
	13	C	9	0	0	9
	13	W	6	0	0	6
1998	16	-	1	0	0	1
2000	16	E	0	nr	nr	nr
	16	C	3	0	0	3
	16	W	0	nr	nr	nr
1998	18	-	4	0	0	4
2000	18	E, C, W	0	nr	nr	nr

Samples collected within rows planted with a potato crop (C) and laterally between rows (E, W)
nr = no isolates recovered

Non-segregating F1 populations were produced by the S, I and R isolates. The EC_{50}s of F1s obtained from S parental isolates all fell into the "sensitive" range with a slight shift toward increased sensitivity while the progeny of the R isolates were found to be "resistant", ranging in a normal distribution around the EC_{50}s of the respective parental isolates. Greater variability was observed in the F1 populations obtained from I parental types with a shift toward resistance occurring in the populations derived from the isolates with the higher EC values (Figure 2). The progeny generally fit a normal frequency distribution but with significant number of "outliers". One R isolate was produced by parental isolate 172-4. Five S isolates and 1 R isolate were identified in the F1 population from isolate 228-3.

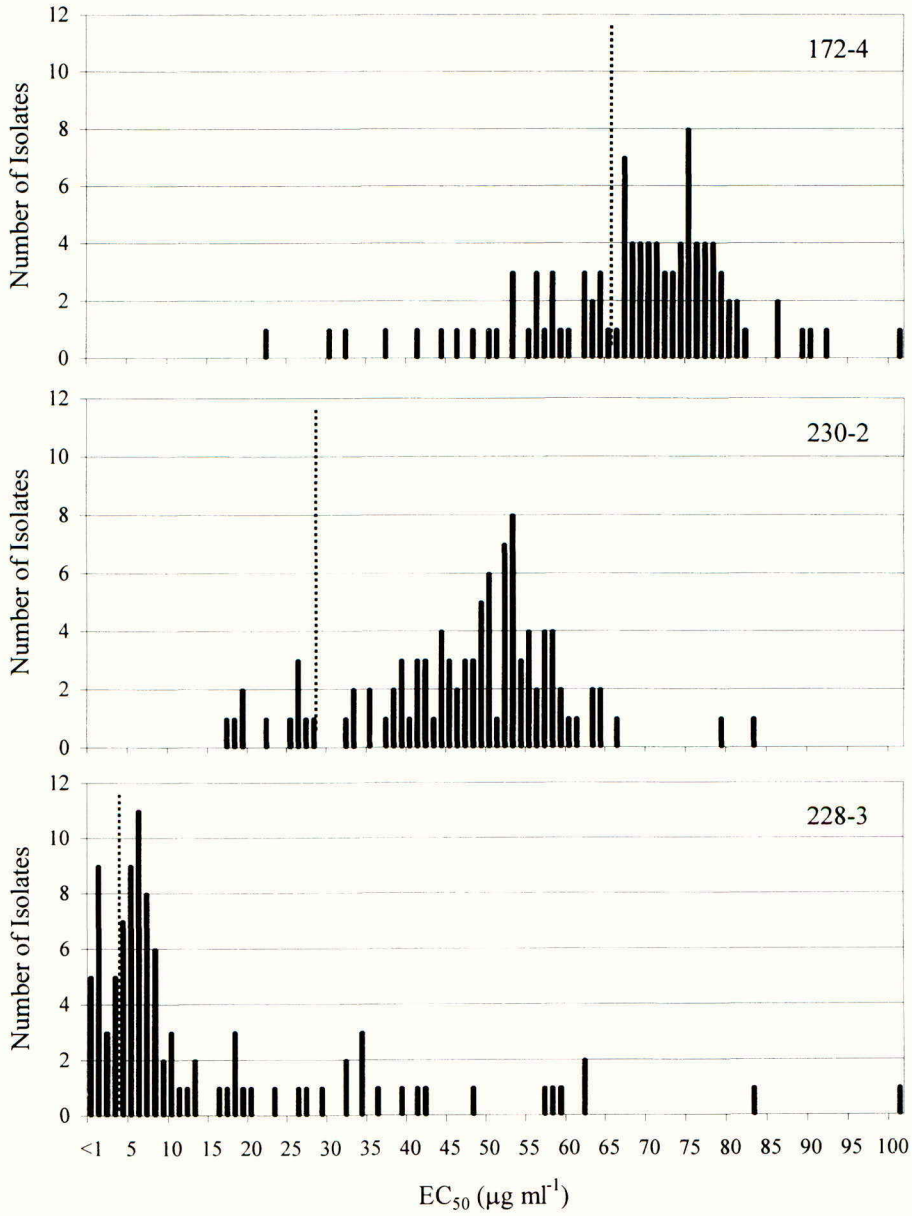

Figure 2. Frequency distribution of mefenoxam sensitivity in F1 populations of *P. erythroseptica* derived from isolates with intermediate responses to mefenoxam. (EC_{50}s of parental isolates are indicated by broken line)

Data presented here confirms the presence of mefenoxam-resistant isolates of *P. erythroseptica* in the potato-growing regions of Maine, Idaho and Minnesota in the United States. The fact that isolates with an "intermediate" response to the fungicide only were found in these areas suggests that the population there may be in a dynamic state of flux. Displacement of these isolates should readily occur with intense selection pressure provided by mefenoxam fungicide use. The results obtained with the soil isolates and F1 populations appear to corroborate this, as do results from prior work dealing with the biological significance of resistance (Taylor *et al.*, 2002b). In that study we found that R isolates and I isolates with EC_{50}s as low as 4.6 μg ml^{-1} were able to overcome the levels of mefenoxam in tubers attained by standard field application practices. These factors should be considered when developing strategies for pink rot and mefenoxam resistance management. To enhance the effectiveness of such programs, further studies are needed to determine temporal distribution and sensitivity of the pathogen in soil when mefenoxam selection pressure is removed. In conjunction, additional work is needed to assess genetic diversity and segregation for mefenoxam resistance in F2 populations of the pathogen.

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Activity of zoxamide against European isolates of *Phytophthora infestans*

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ABSTRACT

Zoxamide is a single-site inhibiting non-systemic fungicide recently registered in Europe for the control of potato late blight. Potato leaf discs sprayed with a range of zoxamide concentrations were used to test the sensitivities of *Phytophthora infestans* isolates never exposed to zoxamide and of isolates obtained from European field trials in nine countries (1997-2000). Isolates varied in sensitivity with minimum inhibitory concentration values from 0.5 to >20 mg/litre. Sensitivity of isolates was not affected by zoxamide treatment and was unrelated to phenylamide resistance. Selected isolates exhibiting a range of *in vivo* sensitivities were tested *in vitro* using poisoned agar plates; all proved very sensitive (EC_{50} values 12-88 µg zoxamide/litre). A scanning microscopy study showed that on the potato leaf surface zoxamide acts after germination, inhibiting further development of hyphae and preventing production of sporangiophores and sporangia.

INTRODUCTION

The benzamide fungicide zoxamide (RH-7281, Zoxium) was developed by Rohm & Haas (now Dow Agrosciences LLC) and has recently been registered in Europe as 'Electis', a co-formulation with mancozeb, for the control of potato late blight, caused by *Phytophthora infestans*. It is specifically active against Oomycete plant pathogens (Egan *et al.*, 1998) and acts by binding covalently to fungal β-tubulin (Young & Slawecki, 2001), inhibiting nuclear division. Zoxamide + mancozeb formulations have shown rapid rainfastness and good protectant activity against late blight in field trials (Bradshaw & Schepers, 2000).

EU registration of new pesticides requires information on the potential for the occurrence and development of resistance in pathogen populations. In support of this, a study of the sensitivity of European isolates of *P. infestans* to zoxamide was instigated. Since the uptake of zoxamide into potato leaf discs is insufficient for a floating disc test (used for phenylamides) to be appropriate, a protocol was developed using leaf discs sprayed with a series of zoxamide concentrations, inoculated with *P. infestans* and assessed for sporulating growth. This was used to test 33 laboratory isolates from sites never exposed to this fungicide and 136 isolates from field trials in nine countries over a four year period, some from zoxamide-treated plots. Selected isolates were also tested *in vitro* using a poisoned agar plate method. In addition, the effect of zoxamide on the development of *P. infestans* from zoospores and directly germinating sporangia on inoculated potato leaves was investigated by scanning electron microscopy.

MATERIALS AND METHODS

Potato plants

Healthy glasshouse-grown potted potato plants (blight-susceptible cultivars lacking R-genes) from high grade seed tubers were used to provide leaf material for inoculation. Leaflets were harvested from plants immediately before use.

Sources of isolates of *Phytophthora infestans* used in the study

Nineteen isolates of *P. infestans* obtained from potato foliage in five European countries during 1996-97 were supplied from a culture collection. In addition, 14 isolates were selected from the Northern Ireland *P. infestans* collection (13 from potato foliage and one from a tuber). These 'laboratory' isolates were all from locations where zoxamide had never been used.

Samples of infected potato foliage, potato tubers or, in one case, tomato fruit and foliage were supplied by Dow AgroSciences from European zoxamide field trial sites, 1997-2000. On receipt, these were incubated under high humidity to induce sporulation. Sporangial/zoospore suspensions were used to inoculate detached healthy leaflets from glasshouse-grown potato plants and isolates maintained on these with weekly transfers (Cooke, 1986). Where possible, isolates were established from both zoxamide-treated and non-zoxamide-treated plots within the same trial. Isolates were subsequently transferred into axenic culture.

Determination of mating type and phenylamide resistance

Mating type was determined by pairing isolates with tester isolates of known mating type on agar plates and monitoring oospore formation. Sporangial suspensions, prepared from isolates growing on detached leaflets, were tested for phenylamide resistance using the floating leaf disc technique (Cooke, 1986) on 100 and 2 mg metalaxyl/litre.

Zoxamide sensitivity – *in vivo* test

Stock solutions, prepared from technical grade zoxamide (92%) dissolved in acetone, were diluted with water (1:99) immediately before spray application to give 20, 10, 5, 1, 0.5 and 0 mg zoxamide/litre. A 40 mg zoxamide/litre concentration was used in a few tests where isolates grew on discs treated with 20 mg/l. The abaxial surfaces of discs (24 mm diameter), cut from potato leaflets, were sprayed with the zoxamide solutions, allowed to dry, then placed abaxial side up on damp filter paper in Petri dishes. Each isolate was tested using five replicate discs for each of five concentrations of zoxamide and a control (1% aqueous acetone). Discs were inoculated with the appropriate sporangial suspension (20 µl, *c.* 5 x 10^4 sporangia/ml) and incubated at 15°C with illumination. After 7 days, discs were assessed for sporulating growth of *P. infestans*. Results were expressed as the minimum inhibitory concentration (MIC), defined as the concentration which inhibited growth on at least four of the five replicate discs. Where possible, isolates were tested at least twice to confirm results. A common isolate (RH1b from the 1997 field samples) was included as a standard.

Zoxamide sensitivity – *in vitro* test

An *in vitro* poisoned plate protocol (derived from one supplied by Dr David Young, Dow AgroSciences) was used to test selected isolates. Aliquots of solutions of technical grade zoxamide in acetone were added to agar to give final concentrations of 0, 0.98, 3.9, 15.6 and 62.5 µg zoxamide/litre. Petri plates containing the treated agar were inoculated centrally with mycelial plugs of the test isolates (three replicate plates/concentration including the control), incubated at 15°C and the diameters of the mycelial growth zones measured after 14 days. EC_{50} values were derived from log-probability plots.

Preparation of fungicide-treated leaf material for scanning electron microscopy (SEM)

Zoxamide suspensions (270 mg/litre; equivalent to 150 g a.i./ha high volume) prepared from 'RH-7281 2F' (240 g/litre) were sprayed onto the adaxial surfaces of potato leaflets. The leaflets were allowed to dry, then placed adaxial side up in humid boxes. For direct germination, sporangial suspensions were prepared in sterile distilled water at ambient temperature (*c.* 20°C) and used immediately. For indirect germination, sporangial suspensions were prepared in refrigerated water and chilled (4°C, 2 h) to stimulate zoospore release. Inoculated leaflets were incubated (12 h light, 12 h dark) at 15°C for indirect germination and at 20°C for direct germination. After 24-96 hours, small pieces of leaflet were excised from the vicinity of the inoculum drops, fixed to a copper stub, rapidly frozen in liquid nitrogen slushed in an argon atmosphere and sputter-coated with gold. The samples were transferred under vacuum to a JEOL JCM-35CF SEM equipped with a cryo-stage for microscopic examination.

RESULTS

Isolates of *Phytophthora infestans*

In addition to the 33 laboratory isolates, 136 isolates from nine countries (Belgium, France, Germany, Italy, the Netherlands, Poland, Portugal, Spain and the UK) were obtained from the field trial samples and tested for zoxamide sensitivity. The numbers of isolates obtained in the years 1997, 1998, 1999 and 2000 were 25, 55, 39 and 17, respectively. The field isolates were all were derived from infected potato foliage, except for one from a potato tuber and two from a tomato sample from Italy (one from foliage and one from a fruit).

Zoxamide sensitivity *in vivo*

The 169 isolates tested using the *in vivo* technique varied in their sensitivity to zoxamide with mean MIC values from 0.5 to >20 mg/litre (Figure 1). Isolates derived from samples received and tested late in the season tended to give higher MIC values. This was not due to an inherently lower sensitivity to zoxamide, since the standard isolate also gave higher MIC values in these tests. The response appeared to be affected by the physiological condition of the leaf material, which, although derived from healthy, glasshouse-grown plants, varied due to environmental and seasonal factors, particularly when tests were performed outside the normal growing season.

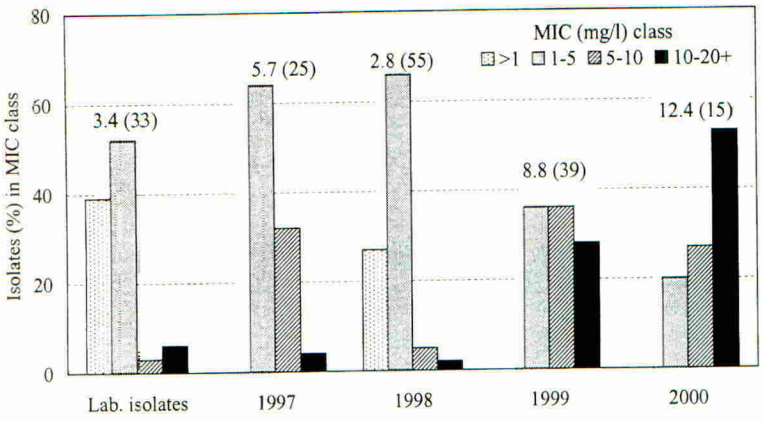

Figure 1. Sensitivity to zoxamide of *Phytophthora infestans* isolates from different years. The mean MIC value (mg/l) is shown above each group of bars followed by the number of isolates tested (in parentheses).

Zoxamide sensitivity *in vivo*: effect of treatment, phenylamide resistance and mating type

Of the 169 isolates tested on leaf discs, 39 were from zoxamide-treated plots. These isolates had a very similar range of sensitivities to those from non-zoxamide-treated plots or crops (Figure 2). In phenylamide resistance tests, 46% of isolates proved to be phenylamide-sensitive, 33% -intermediate and 21% -resistant. Almost all countries yielded a mixture of sensitive, intermediate and resistant isolates, except that all eight isolates from Italy were sensitive. There was no significant association between phenylamide resistance and zoxamide sensitivity. The mean MIC values for the phenylamide-sensitive, -intermediate and –resistant isolates were 5.9, 5.5 and 4.1 mg zoxamide/litre, respectively. Almost all the isolates proved to be of the A1 mating type, only 7 were A2 (from the Netherlands, Poland and the UK), so no conclusions could be drawn regarding zoxamide sensitivity and mating type.

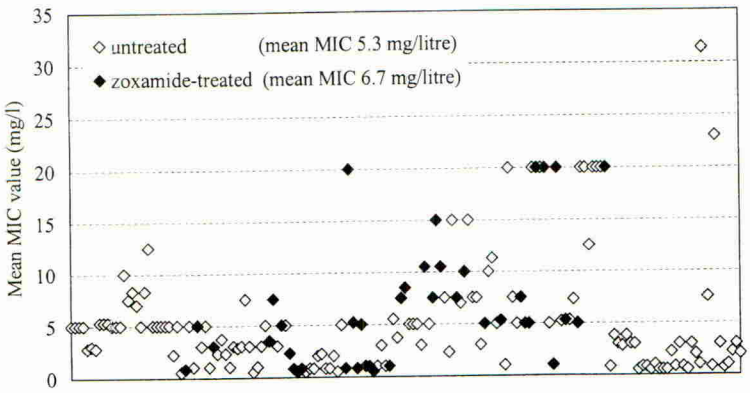

Figure 2. Effect of treatment on sensitivity to zoxamide of *Phytophthora infestans* isolates.

Zoxamide sensitivity *in vivo* and *in vitro*

In vitro sensitivity tests on 14 of the year 2000 field isolates showed that all were very sensitive to zoxamide. Mean EC_{50} values ranged from 25 – 88 µg/litre. Comparison of mean EC_{50} values with mean MIC values, where both were available (Figure 3), showed a lack of correlation between the *in vivo* and *in vitro* sensitivities.

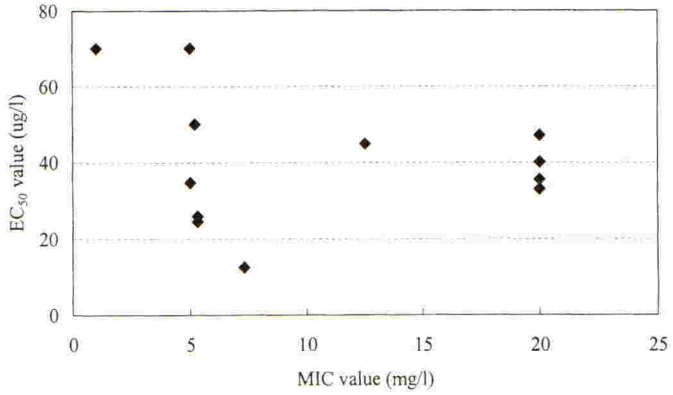

Figure 3. Comparison of *in vitro* (EC_{50}) and *in vivo* (MIC) sensitivity to zoxamide of *Phytophthora infestans* isolates.

Effect of zoxamide on infection of potato leaves by *Phytophthora infestans* (SEM study)

Zoospores germinated on zoxamide-sprayed leaf surfaces, but development was subsequently inhibited (Figure 4). No sporangiophores or new sporangia were observed on zoxamide-treated leaves whereas on untreated leaves inoculated with zoospores these had developed within 96 hours of inoculation. Infection by direct sporangial germination was similarly inhibited.

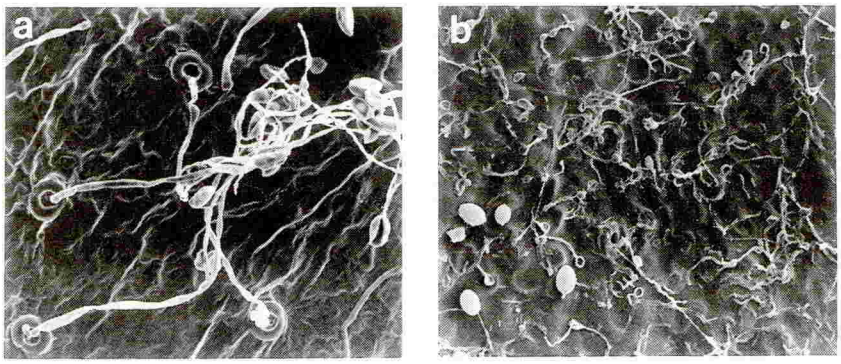

Figure 4. Development of *Phytophthora infestans* on the adaxial potato leaf surface 96 h after inoculation with a sporangial/zoospore suspension (indirect sporangial germination); a) untreated: zoospores have infected the leaf and sporangiophores bearing the new generation of sporangia are emerging from stomata; b) zoxamide-sprayed: after initial germination further development has ceased and debris of sporangia and zoospores from the inoculum are visible on the surface.

DISCUSSION

In vivo techniques for assessing fungicide sensitivity provide a more realistic measure than *in vitro* techniques, but are intrinsically more variable. This applies particularly to non-systemics such as zoxamide, where uniform deposition on the leaf surface is crucial. The use of potato foliage introduces additional variation, since it is strongly affected by season and growing conditions, but pathogenic specialisation within *P. infestans* makes it desirable to use this host plant where potato late blight is the target disease. Consequently, variation over time in results of the *in vivo* zoxamide sensitivity tests complicated interpretation. In tests late in the growing season, all isolates, including the standard, appeared less sensitive, but repeat tests early each succeeding year indicated greater sensitivity as did *in vitro* tests on selected year 2000 isolates (EC_{50} values <100 µg/litre). Despite variation between tests, some European isolates of *P. infestans* were consistently more sensitive than others, probably due to natural variation within the pathogen population, since it was as great among laboratory isolates never exposed to zoxamide in the field as among isolates from sites where zoxamide was applied. Although the present study failed to identify any isolates which consistently exhibited reduced sensitivity, the possibility that such strains occur at low frequency cannot be excluded. Moreover, the divergence between *P. infestans* populations now causing late blight makes it desirable that sensitivity studies should be extended e.g. to North America. Laboratory studies have indicated that the risk of development of resistance to zoxamide is much lower than for metalaxyl, but, as a single site inhibitor, an anti-resistance strategy should be adopted (Young *et al.*, 2001). Formulating zoxamide with mancozeb is one such strategy and scanning electron microscopy has shown that the activities of mancozeb and zoxamide are complementary, mancozeb killing spores before germination and zoxamide inhibiting their subsequent development.

ACKNOWLEDGEMENTS

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Effect of dose rate and mixture on selection for reduced sensitivity to triazole fungicides in *Mycosphaerella graminicola*

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ABSTRACT

The effects of varying doses of fungicides alone or in mixtures, on selection for triazole resistance were examined under field conditions. The triazole fungicide fluquinconazole with the strobilurin fungicide azoxystrobin as a mixture partner were the fungicides tested. Inoculated wheat plots with a known ratio of relatively sensitive to resistant isolates of the leaf blotch fungus *Mycosphaerella graminicola* were sprayed with fungicides and sampled some weeks later. Selection for fluquinconazole resistance was greater in proportion to the dose up to one-half of the full rate. Higher doses were not tested. There was no measured change in selection for triazole resistance due to the addition of the mixture partner azoxystrobin.

INTRODUCTION

Sterol Demethylation Inhibitors (DMI's) were introduced in the late 1960's as agricultural fungicides. Nowadays DMI's and especially azoles are one of the most commercially valuable group of fungicides. Although the risk of resistance to DMI's was initially considered to be low to moderate, several reports of reduced sensitivity or even resistance to DMI's have been published (De Waard, *et al.*, 1986, Leroux, *et al.*, 1991). To overcome resistance development, the control of fungicide doses and mixing of fungicides with different mode of action have been proposed. However the theories and experimental data for how the fungicide doses affect the strength of selection are inconsistent. The main guidelines from FRAC are to limit the number of applications, to use the full-recommended dose and to mix fungicides with different modes of action. This advice is based on the hypothesis that a high dose will kill isolates of all sensitivities and therefore minimise the build-up of the resistant genotypes. A contrasting hypothesis is that a reduced dose might decrease the evolution of resistance by allowing more sensitive isolates to survive and slow down the rate of change within the population.

To understand better how the risk for resistance can be reduced it is important to distinguish between control and selection. Control is determined by the absolute level of kill achieved by a fungicide whereas selection is determined entirely by the relative level of kill of the resistant or sensitive populations (Shaw, 2000).

The aim of the work reported here was to: (1) establish the relationship between dose and selection by the triazole fluquinconazole in the field (2) to test the effect of a strobilurin mixture partner on selection.

MATERIALS AND METHODS

A field trial was established on 11th May 2001 at Shinfield Field Station (University of Reading). Wheat plots of the winter cultivar Riband, with a NIAB rating for resistance to *Mycosphaerella graminicola* of 3, were sown in a randomized complete block design with four replicates in each block. Plots were 6×6 m and barley guards (2 m) were sown around each plot in order to separate the plots and reduce movement of isolates from one plot to another (Metcalfe *et al.*, 2000). There was no other chemical control, so as to avoid any interaction with the experimental substances.

The eight *M. graminicola* isolates used for inoculation were chosen to fall at the extremes of the sensitivity range to fluquinconazole (Table 1). Inoculated plots were sprayed with a mixture of all the isolates at GS – 13 on June 2nd. The ratio of sensitive:resistant isolates was 1:1 and all the plots received equal proportions of each isolate. A spore suspension was prepared from *Septoria tritici* cultures, the volume of the suspension was adjusted to 2×10^7 conidia ml^{-1} and the inoculum was sprayed in 2000 litres of water ha^{-1} with a CO_2 pressurised knapsack sprayer with a 2 m hand held boom. Four plots were left untreated to monitor natural disease.

Table 1. The 8 isolates of *Mycosphaerella graminicola* used to inoculate the wheat plots and their sensitivity to fluquinconazole (FQ) and azoxystrobin (AZ)

Code	$\log_{10}$(EC50/mg l^{-1})		Code	$\log_{10}$(EC50/mg l^{-1})	
	FQ	AZ		FQ	AZ
ST-43	-2.5	-1.0	ST-53	-1.2	-1.0
ST-34	-2.4	-1.3	ST-12	-1.1	-1.5
ST-42	-2.3	-1.3	ST-20	-1.1	-1.3
ST-05	-1.5	-0.99	ST-55	-0.7	-0.8

The fungicides tested were commercial formulations of fluquinconazole and azoxystrobin (Table 2). All fungicide treatments (Table 3) were applied at GS –37 on July 20th in 500 litres of water ha^{-1} with a CO_2 pressurised knapsack sprayer with a 2 m hand held boom.

Table 2. The fungicides used in the field experiment along with some of their details. RR the recommended from the company rate for spraying.

Active Ingredient	Group	Formulation	R R	Company
azoxystrobin	Strobilurin	Amistar 250 g L^{-1}, SC	250 g a.i./ha	Syngenta
fluquinconazole	Triazole	Flamenco 100 g L^{-1}, SC	125 g a.i./ha	Bayer Crop Science

Table 3. Treatments used in the field trial. The recommended rate (RR) for the fluquinconazole (FQ) is 1.25 L/ha and for azoxystrobin (AZ) is 1.0 L/ha

Code	Treatment	grams of active ingredient (a.i.) per hectare
A	Control 1 (for monitoring of the natural disease)	-
B	Control 2 (Septoria only)	-
C	FQ 1/2 of the RR	62 g of fluquinconazole
D	FQ 1/4 of the RR	31 g of fluquinconazole
E	FQ 1/8 of the RR	16 g of fluquinconazole
F	AZ 1/8 of the RR	31.3 g of azoxystrobin
G	FQ (1/2)+AZ (1/8) of the RR	62 g of fluquinconazole +31.3 g of azoxystrobin
H	FQ (1/4)+AZ (1/8) of the RR	31 g of fluquinconazole +31.3 of g azoxystrobin
I	FQ (1/8)+AZ (1/8) of the RR	16 g of fluquinconazole +31.3 of g azoxystrobin

The first sampling was done before the fungicide applications on 18th of July at growth stage GS –36. Samples of infected leaves with *M. graminicola* lesions were randomly taken from all the plots.

The second sampling was done after the appearance of the septoria lesions on the top leaf layers and within six weeks of fungicide application. From each plot 25 plants were collected with the top 4 leaves visibly infected with typical pycnidia of *M. graminicola*. The three leaves below the flag leaf were collected and frozen. Flag leaves were not infected if present. Plots were irrigated as necessary to avoid leaf senescence due to water stress.

The technique used to identify the isolates was described by Metcalfe *et al.* (2000). Leaves with visible pycnidia on them were surface sterilized with 1% sodium-hypochlorite solution. Leaves were then placed in sterilized plastic sandwich boxes on distilled water agar and left to incubate in the dark for 24 hours at 18 C. After 24 hours the cirri were visible and a single cirrus from each lesion was picked up randomly and suspended in 0.5 ml of sterile water. From this suspension 45 µL was used to inoculate corresponding wells in two microtitre plates containing glucose peptone broth, one amended with a discriminating dose of fluquinconazole. Growth in the unamended plate only indicated as sensitive; growth in both as resistant.

RESULTS

In the plots that were left uninoculated nor sprayed it was impossible to find any diseased leaves, so that any external immigration of the pathogen was very slight. A very high incidence of gout fly infection of the wheat apices meant that almost no ears emerged and the plants remained rather short; leaf senescence appeared to be greatly delayed.

Sensitivity of population before spaying

Before the fungicide application all plots had the same frequency of R isolates, which was about 45% (X^2_{30}=32.3).

Sensitivity of population after spraying – single fungicides

In the plots treated with fluquinconazole only, selection for resistance was greater in proportion to the dose. Increasing dose from 1/8 to 1/4 or 1/2 significantly increased selection for triazole resistance (Figure 1). The strongest selection occurred in the plots that received the highest dose (1/2 of the recommended dose).

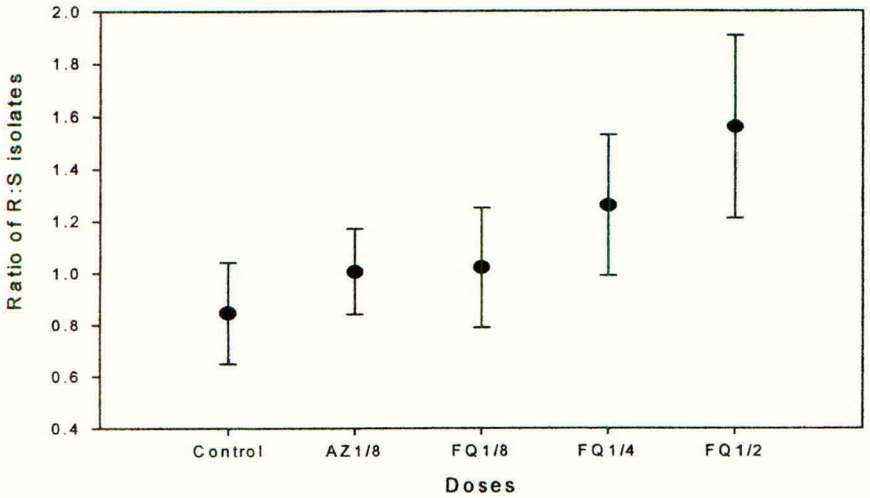

Figure 1. Effects of different doses of fluquinconazole (FQ) and azoxystrobin (AZ) on the ratio of Resistant to Sensitive (R:S) isolates of *Mycosphaerella graminicola*.

Fungicide sensitivity after spaying – mixtures

When a mixture of fluquinconazole and azoxystrobin was applied there was no significant change (X_3^2=1.02) in selection for resistance due to the addition of the mixture partner azoxystrobin (Table 4). The reduction at the highest dose of fluquinconazole is not significant.

Table 4. The ratio of Resistant to Sensitive isolates (R:S) when fluquinconazole (FQ) sprayed alone and with 1/8 of the recommended rate of azoxystrobin (AZ).

Doses of (FQ)	Ratio of Resistant to Sensitive Isolates	
	FQ	FQ+AZ
1/8	1.1	1.0
1/4	1.2	1.3
1/2	1.7	1.4

DISCUSSION

This experiment suggests that application of fluquinconazole at reduced doses may cause significantly decrease selection for triazole resistance. This agrees with the results of (Metcalfe *et al.*, 2000), and Peever *et al.*, (1994), but extends them to one-half full recommended dose. Peever *et al* included a full and double dose of propiconazole in their work of *Stagonospora nodorum*, but did not detect selection probably because of small sample sizes. The key question is whether the results here can be extrapolated to full doses in realistic field settings. Shaw (2000) showed using a mathematical model that if populations after spraying included survivors from unsprayed refugia, there could be a critical dose, below which selection reduced by reducing dose but above which selection was reduced by increasing dose. It is possible that full recommended doses of triazoles are above this threshold, but without doing the experiment it hard to be certain and there is no a priori reason why they should be. The problem of including a full dose in experiments of the design made here is the scale of sampling and plots needed to find sufficient lesions in plots treated at full rate in years not favorable to *Septoria tritici*. Our resource was inadequate to this task.

The measurements made focused only on the effect of a single spray. Clearly reducing the dose but increasing the number of applications would have a cumulative effect on the total selection occurring, which might be worse than the effect of a single large dose.

There was no significant difference in selection when azoxystrobin was mixed with fluquinconazole. These fungicides belong to different chemical groups and have independent modes of action as is usually recommended for effective mixtures. There is no consensus as to the effect of fungicide mixtures on the evolution of the resistance and at the best way to use two active ingredients. Different mathematical models have been developed to provide answer of what is the best way to use fungicide in mixtures. Kable *et al* (1980) support that the alternation of fungicides is the preferable way of combating resistance and Skylakakis (1981) agreed that mixtures would always slow selection for resistance except possibly when the latent periods of the pathogen were very short. Shaw (1989) argued that the effect of combining fungicides in a mixture on the strength of selection depended of details of the interaction between the components. In the simplest situation no effect was to be expected. He pointed out the need to find ways to measure synergism between fungicides used in mixtures based on evolution of resistance, not on the control that they provide. The findings of our experiment support the idea that a mixture of independent acting fungicides does not slow down selection. However, the results in not conclusive and might in any cause apply only to a specific fungicides used. Further work has to be done with different combinations of fungicide mixtures.

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