# POSTER SESSION 4D

# RESISTANCE OF PESTS AND PATHOGENS TO PESTICIDES

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# *In planta* genotyping of *Erysiphe graminis* f. sp. *tritici* isolates for strobilurin-resistance using a fluorometric allele-specific PCR assay

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## ABSTRACT

Three strobilurin-resistant isolates of *Erysiphe graminis* f. sp. *tritici* from Germany and the UK were 100-fold less sensitive to azoxystrobin than sensitive isolates. A 675 base pair (bp) fragment of the mitochondrial cytochrome *b* gene was sequenced and a guanine to cytosine (G-to-C) change for one nucleotide was identified. The mutation resulted in the substitution of a glycine for an alanine residue at codon 143 (G143A) in the cytochrome *b* of resistant isolates. Allele-specific primers were designed to detect this point mutation in infected leaves by polymerase chain reaction (PCR) linked to dsDNA-specific fluorescence. The resistant A143 allele can be detected amongst sensitive G143 alleles at a frequency below 1:1000. Eight isolates were tested and no false positives were found, although the sensitive G143 allele could be detected, albeit in low amounts, in strobilurin-resistant isolates. This suggests that a mixed population of strobilurin-sensitive and resistant mitochondria could be present in these isolates.

## INTRODUCTION

Powdery mildew caused by the obligate biotrophic fungus *Erysiphe (Blumeria) graminis* f. sp. *tritici*, is a serious threat in all European wheat-growing areas. Good farming practices, including crop rotation and resistant cultivars can reduce infection, but fungicides are the main control for powdery mildew epidemics. Although many fungicides are available to control the disease, rapid development of resistance is a major problem (Hollomon & Wheeler, in press). Decreased sensitivities have been reported to hydroxypyrimidines, morpholines, sterol 14$\alpha$-demethylation inhibitors (DMIs) and, recently, strobilurins (Anonymous, 2000). Fungicide resistance can evolve by decreased uptake, increased efflux, metabolic detoxification or by compensation and alteration of the target site. Overall, the resistance risk is generally high for single-site inhibitors, whilst polygenic-based resistance evolves more slowly and rarely causes product failures. The rapid spread of strobilurin resistance from Northern-Germany in 1998 and elsewhere in Europe within two years indicates the involvement of a single-step target site change. Several point mutations in two different regions of the mitochondrial cytochrome *b* gene can confer resistance to strobilurins and related compounds (STARs) in mutants of *Saccharomyces cerevisiae* (Di Rago *et al.*, 1989). Allele-specific PCR (ASPCR) (Délye *et al.*, 1997), PCR-Restriction Fragment Length Polymorphism (RFLP) (Luck & Gillings, 1995) and PCR linked with allele-specific oligonucleotide probes (Koenraadt & Jones, 1992) have been used to detect point mutations conferring resistance to DMI and benzimidazole fungicides.
Our objective was to test the hypothesis that a particular amino acid substitution in the cytochrome *b* protein was correlated with strobilurin-resistance in *E. graminis* f. sp. *tritici*, and to develop a PCR-based test to detect, quantify and monitor strobilurin resistance.

## MATERIALS AND METHODS

### Fungal isolates

All *E. graminis* f. sp. *tritici* isolates were purified as single-colony isolates (Table 1). Isolates were maintained at 17 °C on untreated leaf pieces (cultivar Riband) on 0.5 % (w/v) water agar containing benzimidazole (50 ppm) in plastic boxes, and subcultured at 10-day intervals.

Table 1. *Erysiphe graminis* f. sp. *tritici* isolates tested.

| Isolate designation | Origin | Year | Azoxystrobin sensitivity * |
|---|---|---|---|
| W26 | SW-England | 1987 | 0.05 |
| JAS501 | N-Germany | 1998 | 5.0 |
| JAS506 | N-Germany | 1998 | 5.0 |
| DE60.3 | NE-England | 1999 | 0.05 |
| DE60.4 | NE-England | 1999 | 0.05 |
| DE74.1 | S-England | 1999 | 0.05 |
| DE75.2 | S-England | 1999 | 0.05 |
| GBlet1 | UK | 1999 | >5.0 |

* For each isolate, the minimal concentration ($\mu g\ ml^{-1}$) to inhibit all infection is given.

### Strobilurin sensitivity assays

Leaf pieces were floated on 10-fold dilutions of azoxystrobin (0.0005 to 5.0 $\mu g\ ml^{-1}$) for 16 h at 17 °C. Leaf pieces were inoculated by blowing viable conidia into a settling tower, and initially kept in darkness for 6 h at room temperature to synchronise spore germination. After 72 h incubation under white light at 17 °C, leaves were cleared and stained with 0.05% trypan blue in glycerol, lactic acid and water (1:1:1). The number of colonies formed on each leaf was counted under a microscope, and final counts of treated leaves were compared with those of fungicide-untreated leaves to determine the minimum concentration to inhibit all infection.

### DNA extraction

DNA extraction from conidia and from infected and healthy wheat leaves, and, subsequent measurement of DNA concentrations was done according to Fraaije *et al.* (1999).

### Cloning and sequencing part of the cytochrome b gene

Using primer set CBF1/CBR3 (see Table 2), a 675 bp fragment of the cytochrome b gene from strobilurin-sensitive and resistant isolates was amplified. The PCR fragment codes for the amino acid codon regions 127-153 and 255-276 of the cytochrome b where point mutations linked to strobilurin resistance have been reported for various fungi (Zheng & Köller, 1997). After PCR, excess primers were removed with the High Pure PCR Product Purification kit and the PCR products were ligated directly into the pGEM-T easy vector and subsequently sequenced according to standard protocols.

Table 2. Primers for amplifying cytochrome *b* gene sequences of *E. graminis* f. sp. *tritici*

| Primer designation | Sequence (5'-3') | Amino acid position * |
|---|---|---|
| CBF1 | TATTATGAGAGATGTAAATAATGG | 68-74 |
| F1 | ATACGTTACATACACTCAAACACA | 78-85 |
| 143CF | CAGATGAGCCACTGGGC | 138-142 |
| 143GF | CAGATGAGCCACTGGGG | 138-142 |
| CBR3 | CCTAATAATTTATTAGGTATAGATCTTA | 284-291 |
| R4 | TAATATTGCATAGAAGGGCAG | 277-283 |
| R5 | ACTCCGGTACAATAGCAGCC | 268-273 |

* Cytochrome *b* of *Venturia inaequalis* as reference (Zheng & Köller, 1997)

**PCR standard protocol**

Standard PCR was carried out in a Biometra T3 thermocycler with 1.25 units of Red Hot DNA polymerase using 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl (pH 9.0), 0.01% Tween-20, 1.5 mM $MgCl_2$, 125 µM of each dNTP, 0.5 µM of each primer, 100 ng of template DNA in a final volume of 100 µl. Conditions to amplify part of the cytochrome *b* gene were 94°C for 3.5 min, followed by 40 cycles at 94°C for 30s, 50 or 55°C for 1 min and 72°C for 1.5 min. The PCR was terminated with a DNA extension at 72°C for 8.5 min. PCR products were visualised under UV light after electrophoresis in ethidium bromide-stained agarose gels.

**Strobilurin-resistance genotyping with a fluorimetric allele-specific PCR assay**

Primers were used that preferentially amplified one allele over another by matching the desired allele but mismatching the other allele at, or close to, the 3' end of the primer (Sarkar *et al.*, 1990). The allele-specific PCR protocol was the same as the standard protocol, except that 0.2 µM of each primer, 0.5 unit Red Hot DNA polymerase, and 200 ng template DNA was used per reaction of 40 µl. DNA template was obtained by extracting DNA from infected and uninfected leaves. The conditions were 94°C for 3.5 min, followed by 45 cycles at 94°C for 30s, 55, 57 or 62°C, depending on the primers, for 40s and 72°C for 40s with a final DNA extension at 72°C for 10 min. The presence of PCR products in each reaction was measured with the dsDNA-specific fluorescent dye PicoGreen (Singer *et al.*, 1997) using a LS50B fluorimeter with microtitre plate attachment (Fraaije *et al.*, 1999).

**RESULTS**

**Strobilurin sensitivity testing**

The three strobilurin-resistant isolates, JAS501, JAS506 and GBlet1, were at least 100-fold less sensitive to azoxystrobin than all sensitive isolates tested (Table 1).

**Cytochrome b gene partial cloning and sequencing**

When DNA of strobilurin-sensitive E. *graminis f. sp. tritici* isolates DE60.4 and W26 was tested, primer combination CBF1/CBR3 generated a single product of 675 bp at 55°C. After sequencing all products it was clear that the expected fragment of the target cytochrome b gene of *E. graminis* f. sp. *tritici* was cloned (Figure 1).

Figure 1. Partial amino acid sequence of cytochrome *b* of *E. graminis* f. sp. *tritici* isolates W26 and DE60.4. Strobilurin-resistant isolates JAS501 and JAS506 revealed an alanine (GCT) residue instead of a glycine (GGT) residue at codon 143 (underlined).

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76  WLIRYIHSNTASAFFFLVYLHIGRGYYGSYRAPRTLVWTIGTVIFILMIVTAFLGYVLP
136 YGQMSHWGATVITNLMSAIPWIGQDIVEFLWGGFSVNNATLNRFFALHFVLPFVLA
192 ALALMHLIALHDSAGSGNPLGVSGNYDRLPMAPYFLFKDLITIFLFIIILSMFVFFMSN
251 VLGDSENYVMANPMQTPAAIVPEWYLLPFYAI
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The sequence contained no introns and encoded for 207 amino acids (codon 76-282) of the cytochrome b protein. Comparison with other fungal amino acid sequences of cytochrome b revealed a high identity between *E. graminis* f. sp. *tritici* and *Aspergillus nidulans* (87.0 %), *Podospora anserina* (87.4 %), *Neurospora crassa* (87.9 %) and *Venturia inaequalis* (90.8 %).

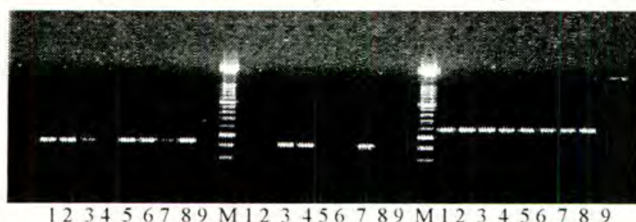
**Identifying point mutation linked with strobilurin-resistance**

To identify point mutations linked with strobilurin resistance in *E. graminis* f. sp. *tritici*, the 675 bp fragment of the cytochrome b gene amplified by CBF1/CBR3 was also sequenced from the strobilurin-resistant isolates JAS501 and JAS506. These sequences revealed a single guanine to cytosine (G-to-C) change at nucleotide 228 of the cloned fragment, which encodes for codon 143 of the cytochrome *b* protein (Figure 1). The point mutation resulted in the substitution of a glycine by an alanine residue at codon 143 (G143A).

**Allele-specific PCR assay for detection of G143A**

Primer sets 143GF/R4 and 143CF/R5 discriminated between the strobilurin-sensitive G143 and resistant A143 alleles of cytochrome b of *E. graminis* f. sp. *tritici*, whilst no fragment was amplified from DNA from uninfected wheat leaves (Figure 2).

Figure 2. Detection of strobilurin-resistance in *E. graminis* f. sp. *tritici* isolates on wheat leaves by allele-specific PCR using primer sets 143GF/R4 (A), 143CF/R5 (B) and F1/R5 (C). Samples (2 µl) were analysed in agarose gel electrophoresis. Lane 1, isolate DE60.3; 2, DE60.4; 3, JAS501; 4, JAS506; 5, DE74.1; 6, DE75.2; 7, Gblet1; 8, W26; 9, uninfected wheat leaf; M, 100 bp DNA ladder

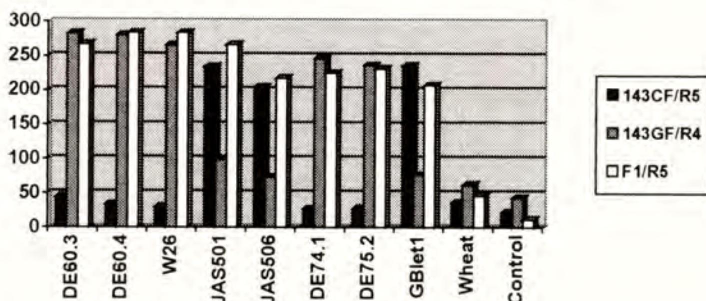



At 57°C, primer set 143GF/R4 amplified a 439 bp product from the strobilurin sensitive isolates, however, a faint band also was observed in the resistant isolates. At 62°C, 143CF/R5 amplified a 409 bp fragment only from the strobilurin-resistant isolates, including isolate GBlet1 recently isolated in the UK. The same point mutation at nucleotide 228 of the cytochrome *b* gene causing G143A was subsequently confirmed in GBlet1 by sequencing. As a positive control, we used primer set F1/R5 at 55°C to amplify a conserved 599 bp fragment of the cytochrome *b* gene from all isolates.

## Rapid genotyping of isolates for strobilurin resistance

The results obtained with fluorimetry (Figure 3) correlated well with the visualised results of allele-specific PCR. The weak and strong positive fluorescence values for isolates JAS501, JAS506 and GBlet1, obtained with 143GF/R4 and 143CF/R5, respectively, confirmed the results of the agarose gel analysis (Figure 2), suggesting that a mixture of strobilurin-sensitive G143 and resistant A143 alleles may be present in these isolates.

Figure 3. Quantitative analysis of allele-specific PCR products with fluorimetry. PCR samples (2 µl) obtained with different primer sets were analysed in the fluorimetric assay. Fluorescence units on Y-axis.




To check for specificity and sensitivity, DNA of uninfected wheat leaves was spiked with plasmid DNA containing the cloned fragment of the cytochrome b gene of isolates W26 and JAS501. Using amounts of plasmid DNA <200 pg per PCR assay, no false positives were obtained when detecting a particular allele (data not shown). However, the sensitivity of detection of the matching strobilurin-sensitive G143 or resistant C143 allele was almost a million-fold higher than the mismatch, as specific alleles could be detected in plasmid DNA <0.002 pg. When mixing samples of JAS501 and DE60.4 infected leaves, the strobilurin-resistant allele A143 of isolate JAS501 could be detected in the presence of a 1000-fold excess of the sensitive G143 alleles of isolate DE60.4 (data not shown).

## DISCUSSION

After comparing partial cytochrome *b* sequences of strobilurin-sensitive and resistant isolates of *E. graminis* f. sp. *tritici*, an amino acid change (G143A) was found to correlate with resistance. Glycine is found at this position in fungi sensitive to STARs, whilst the larger amino acid alanine is present in the strobilurin-resistant basidiomycete *Mycena galopoda*, and probably causes resistance by affecting the inhibitor binding site (Kraiczy *et al.*, 1996). Recently, G143A has also been associated with strobilurin-resistance in a laboratory-mutant of *V. inaequalis* (Köller, 1999).

The fluorimetric allele-specific PCR assay was applied successfully to screen *E. graminis* f. sp. *tritici* isolates for strobilurin-resistance. However, for resistant isolates, the sensitive G143 allele was also detected, albeit in lower amounts than the resistant A143 allele, indicating the presence of a mixed population of mitochondria within a single cell. Strobilurin-resistant A143 alleles could be detected at a frequency of at least 1:1,000 and this is likely to be sensitive enough to detect a point mutation in a single mitochondrial genome.

DMI fungicides may increase the selection pressure for resistant populations (e.g. Metcalfe *et al.*, 1998). Anti-resistance strategies are therefore important to maintain the efficacy of these fungicides. The newer strobilurin fungicides are systemic protectants with minimal curative activity, consequently the curative action of DMI fungicides will remain crucial for the future successful management of *M. graminicola*.

## METHODS AND MATERIALS

### National monitoring

Over 120 isolates of *M. graminicola* were recovered from leaves taken from samples collected from throughout England & Wales as part of the 1999 MAFF-funded National Winter Wheat Disease Survey. These were tested *in vitro* to determine their sensitivity to the DMI fungicides, cyproconazole, flutriafol, flusilazole, epoxiconazole, bromuconazole, difenoconazole, fluquinconazole and tebuconazole.

### Field trials

In 1998 and 1999, trials were carried out to examine the performance of DMI fungicides applied alone and in mixtures with a strobilurin and their effect on sensitivity of *M. graminicola* populations. At each of three sites, trials consisting of randomised plots of $96m^2$, received three replicates of various treatments at GS 31 and GS 39 (Zadoks *et al.*, 1974). In 1998 and 1999, in Northern Ireland and at ADAS Rosemaund, tebuconazole ('Folicur') and epoxiconazole ('Opus') were each applied at full, 0.75, 0.5 and 0.25 field rates; azoxystrobin ('Amistar') was applied at full and 0.5 field rates; and mixtures of both DMIs at each rate were applied with 0.5 rate azoxystrobin. In 1999 at Long Ashton Research Station (LARS), epoxiconazole and azoxystrobin were each applied at full and 0.5 field rates and mixtures of each rate of epoxiconazole were applied with each rate of azoxystrobin. Five isolates of *M. graminicola* were recovered from each plot prior to the first fungicide application and tested to establish a base-line sensitivity of the population to DMI fungicides. Three to four weeks after the second fungicide application, a maximum of 25 lesions were saved from the top two leaves of each plot to determine whether any shift towards reduced sensitivity had occurred.

All sensitivity testing was carried out using a protocol adapted from Pijls *et al.* (1994). Solutions of the fungicides were made up at 20 times the required concentration in ethanol. Ten microlitres of the fungicide/ethanol solutions were pipetted into the bottom of sterile microtitre plate wells. After the ethanol had evaporated, 200µl of Glucose Peptone Broth containing *M. graminicola* spores was added to the well, diluting the fungicide to the required concentration. After incubation at 18°C in the dark for 6 days, fungal growth was measured using a microtitre plate reader at an absorbance of 405nm. The absorbance above that of the media and spore germination alone was used as an estimate of growth, a higher absorbance value indicating more growth.

Growth curves were generated for each isolate and $EC_{50}$ values were calculated for each fungicide tested. The $EC_{50}$ values were used as an estimation of sensitivity of the *M.*

*graminicola* isolates to each fungicide. Regression analyses were carried out on the $log_{10}$ of the $EC_{50}$ values to determine cross-sensitivity relationships.

## RESULTS

### National Monitoring

Analysis of variance showed significant differences ($p<0.001$) between the national mean $EC_{50}$ of each fungicide to the *M. graminicola* isolates tested. $EC_{50}$ values indicate that, *in vitro*, difenoconazole, epoxiconazole and fluquinconazole were the most active fungicides against *M. graminicola*. The older generation DMI, flutriafol, was the least active fungicide followed by bromuconazole and tebuconazole. Cyproconazole and flusilazole were intermediate in their efficacy (Table 1).

Table 1. $EC_{50}$ values of fungicides tested against *M. graminicola* in 1999.

| Fungicide | $EC_{50}$ value (µg $ml^{-1}$) | | |
|---|---|---|---|
| | minimum | mean | maximum |
| difenoconazole | 0.001 | 0.011 [a] | 0.079 |
| epoxiconazole | 0.004 | 0.023 [a] | 0.125 |
| fluquinconazole | 0.001 | 0.058 [a] | 0.207 |
| flusilazole | 0.027 | 0.113 [b] | 2.039 |
| cyproconazole | 0.026 | 0.125 [b] | 0.836 |
| tebuconazole | 0.034 | 0.253 [c] | 1.071 |
| bromuconazole | 0.024 | 0.274 [c] | 1.794 |
| flutriafol | 0.080 | 0.479 [d] | 3.199 |

$EC_{50}$ values sharing the same letter are not significantly different according to Tukeys pairwise comparisons.

Regression analysis indicated that levels of cross-sensitivity to DMIs varied between the DMI combinations tested. Although $r^2$ values as low as 7% were statistically significant due to the large number of degrees of freedom, values of less than 50% were treated with caution due to the many factors influencing sensitivity of *M. graminicola* populations. Strong correlations were evident between cyproconazole and flusilazole, cyproconazole and epoxiconazole, flusilazole and epoxiconazole and between cyproconazole and bromuconazole (Table 2). There was little correlation between tebuconazole, difenoconazole or fluquinconazole with the other DMIs tested (Table 2).

Table 2. Percentage $r^2$ values indicating cross-sensitivity between isolates screened in 1999.

| Fungicide | cypro-conazole | flutriafol | flusilazole | fluquin-conazole | bromu-conazole | difeno-conazole | tebu-conazole |
|---|---|---|---|---|---|---|---|
| flutriafol | 45.7* | | | | | | |
| flusilazole | 72.6* | 53.3* | | | | | |
| fluquinconazole | 4.0 | 2.6 | 10.8* | | | | |
| bromuconazole | 64.0* | 40.9* | 52.1* | 0.3 | | | |
| difenoconazole | 15.6* | 23.3* | 24.0* | 4.2 | 15.7* | | |
| tebuconazole | 14.9* | 13.4* | 26.8* | 22.7* | 8.2* | 7.0* | |
| epoxiconazole | 71.3* | 55.7* | 75.8* | 2.5 | 57.2* | 28.1* | 16.8* |

* = significant at $p<0.05$

**Field trials**

Although results from the different sites varied, the overall trends were similar. The results of ADAS trials presented are representative of the trends observed in Northern Ireland and at LARS. Despite greater disease pressure in 1999, performances of epoxiconazole alone and all fungicide mixtures were better than in 1998. Due to adverse weather conditions, the first spray on the ADAS trial in 1998 was delayed from GS 31 to GS 33 and the second spray was delayed from GS 39 to GS 59 resulting in poorer disease control than in 1999 (Figure 1).

Performance of all treatments varied between seasons and sites. However, at all sites, all treatments except 0.25 rate tebuconazole, significantly reduced disease levels on the top two leaves during the grain filling stage (GS 71 onwards). Epoxiconazole was the most effective DMI used, controlling disease on the top three leaves at 0.5 rate in 1998 and 0.25 rate in 1999. In comparison, only full rate tebuconazole reduced disease on leaf three.

All rates of DMI when applied in mixture with 0.5 rate azoxystrobin resulted in significantly enhanced disease control and increased longevity of the upper leaves (Figure 1). This was highly significant in leaves 2 and 3 in plots treated with 0.5 rate azoxystrobin in mixture with 0.25 rate epoxiconazole, 0.5 and 0.25 rate tebuconazole, where leaf three was rapidly senescing by the middle of grain filling when treated with these DMIs alone. The increased photosynthetic life of leaves two and three particularly, correlated well with yield and yield benefit.

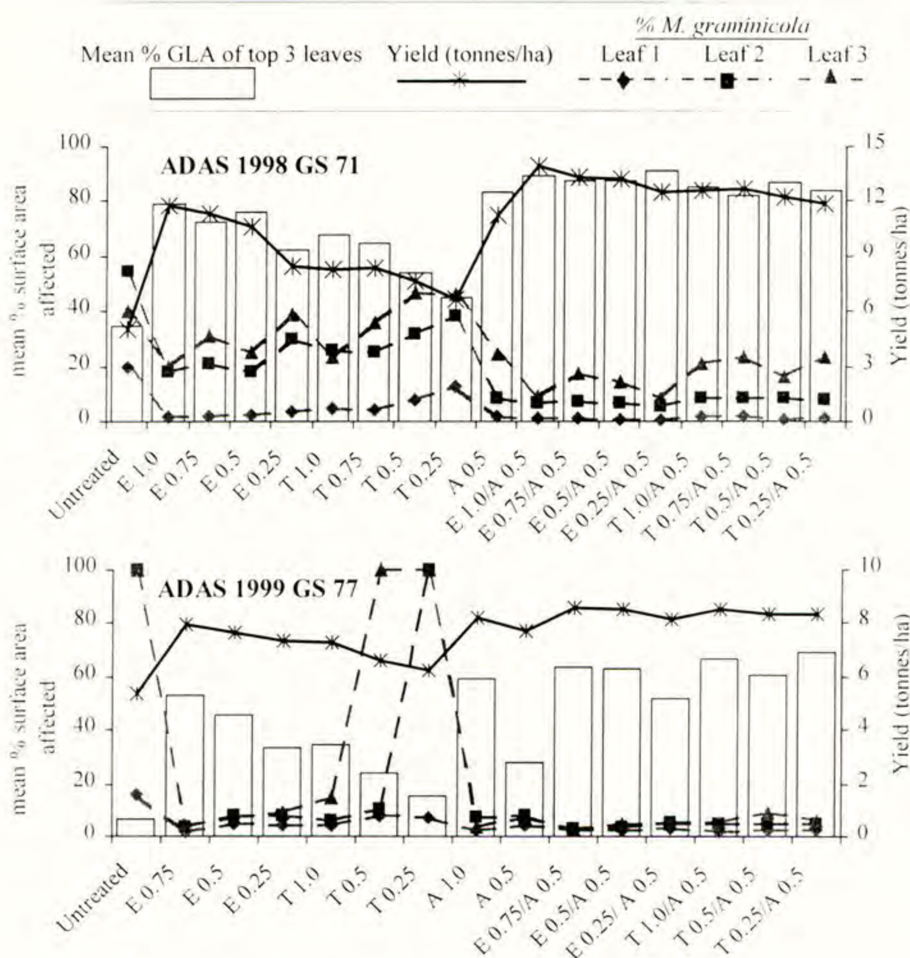



Figure 1. Percentages leaf areas of leaf 1, leaf 2 and leaf 3 affected with *M. graminicola* and the correlations with % green leaf area and yield.

The *in vitro* bioassay of *M. graminicola* isolates taken from the plots prior to fungicide treatment did not reveal a significant shift towards reduced sensitivity to DMI fungicides between seasons at any site. In 1999 at the LARS trial, as in previous seasons, lesions were present but fungicide treatment prevented pycnidial formation or resulted in the production of deformed pycnidia with few pycnidiospores. Epoxiconazole at 0.25 rate and azoxystrobin at 0.5 rate resulted in 2.8% and 13.3% lesions containing pycnidiospores respectively, compared to 47.9% collected from the untreated plots. No pycnidia were detected in plots treated with higher dose rates of the single fungicides or fungicide mixtures. There was also no evidence of a shift in sensitivity in isolates subjected to selection pressure from the fungicides applied within a season. Although low numbers of isolates were consequently recovered, results of sensitivity testing indicated no shift towards

reduced sensitivity to DMI fungicides as a result of fungicide treatment. Similar results were obtained in Northern Ireland: in 1998, isolates obtained from the plots three to four weeks after application of the second spray did not differ in sensitivity from isolates taken from untreated plots (Figure 2). A similar pattern was observed in the 1999 trials, however, as in samples collected from the Long Ashton field trials, very few isolates were obtained from the treated plots.

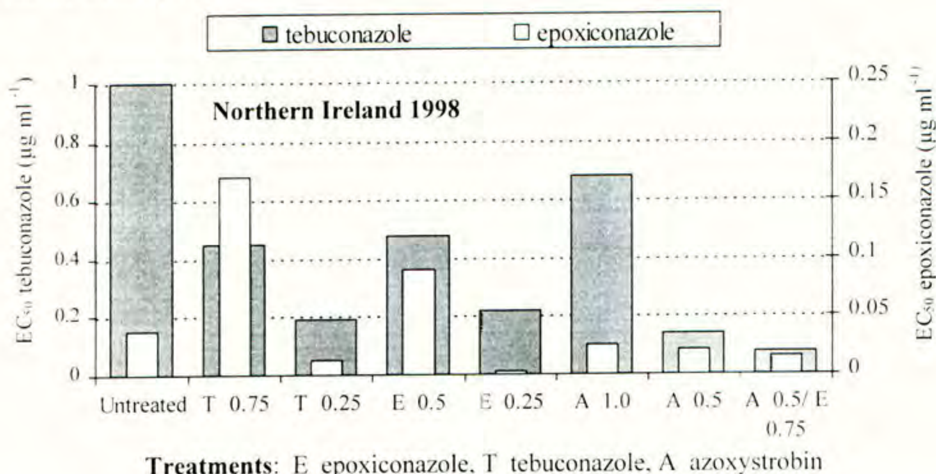



Figure 2. Mean $EC_{50}$ of *M. graminicola* isolates taken from field trial plots in Northern Ireland in 1998

## DISCUSSION

Epoxiconazole was the most widely used active substance applied to wheat, by area treated, in 1998 (Garthwaite & Thomas, 1998). It was also the most effective DMI tested in the field and very effective *in vitro* against *M. graminicola*. National monitoring showed that the $EC_{50}$ recorded for epoxiconazole was ten times lower than that recorded for tebuconazole, confirming field trial results showing epoxiconazole to be the more effective. $EC_{50}$ values for epoxiconazole showed some of the strongest positive correlations with those for other DMIs tested. Good correlations were also evident between flusilazole, cyproconazole, bromuconazole and flutriafol. Cross-resistance usually extends to all fungicides with the same mode of action, although levels can differ between fungicides (Kendall, 1986). If significant shifts towards reduced sensitivity to epoxiconazole were to occur, this could jeopardise the efficacy of many DMI fungicides.

There was no evidence of a shift towards reduced sensitivity between seasons at any trial site. *In vitro* sensitivity testing in 1999 showed that two applications of DMI fungicides or DMI/azoxystrobin mixtures did not significantly affect the sensitivity of the *M. graminicola* populations to DMI fungicides at any of the trial sites. This suggests that selection pressure, exerted by the individual treatments and the frequency of application used in the experiments, was not sufficient to shift the sensitivity of the population from the base-line sensitivity distributions determined between 1990 and 1994 (Hermann & Gisi, 1994) and in

1992, 1994 and 1995 (Turner *et al.*, 1996). This confirms previous reports that shifts in sensitivity of *M. graminicola* to DMI fungicides have not been evident in naturally-occurring populations (Pijls & Shaw, 1997), although shifts have been observed in inoculated trials (Metcalfe *et al.*, 1998). Differences observed in such trials may be due to the increased disease pressure on inoculated plots and the limited diversity of the population introduced.

The increased usage of DMI fungicides and the widespread use of only a few popular DMIs may provide increased selection pressure and thus the potential for a shift towards reduced sensitivity to these fungicides. However, this has not been observed in practice. The curative activity of DMI fungicides to *M. graminicola* is limited to the hyphal colonisation stage of the pathogen life cycle. If fungicide application is delayed, performance is reduced or lost as the disease progresses into the formation of pycnidial initials. The optimal timing of fungicide application at the LARS trial in 1999 resulted in reduced pycnidia and pycnidiospore production as the pathogen was controlled before extensive pycnidia production. If significantly fewer viable spores are produced, fewer spores subjected to selection pressure are released into the background population. The comparative fitness of resistant strains will also determine the influence such strains have on the population, and in the early stages, DMI-resistance is frequently associated with reduced fitness in terms of sporulation, spore germination, growth rate and pathogenicity (Fuchs & de Waard, 1982).

In the field trials, application of optimally timed DMI/azoxystrobin mixtures achieved good control of *M. graminicola* and yield benefits through enhanced longevity of the upper leaves. To prevent the development of fungicide resistance in pathogen populations, it is recommended that mixtures of fungicides with different modes of action are used, thus the practice of applying DMIs in mixtures with strobilurin fungicides, should also contribute to management of sensitivity of *M. graminicola* to the DMI fungicides. The use of mixtures of strobilurin/DMI mixtures has not prevented the development of strobilurin resistance in populations of wheat powdery mildews in Northern Europe (Appel, 1999). However, DMIs are not effective mixture partners in cereal powdery mildew control, since their performance has already been impaired by selection of less sensitive strains. When choosing mixture partners to reduce selection for resistant individuals, it is crucial that both fungicides should have good activity against the target pathogen. This is currently the case with DMIs and azoxystrobin for control of *M. graminicola*, but constant vigilance is required.

## ACKNOWLEDGEMENTS

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## REFERENCES

Appel J (1999). Fungicide resistance and the Fungicide Resistance Action Committee (FRAC). *The Agronomist*, **3**, 10-12.

Fuchs A; de Waard M A (1982). Resistance to ergosterol-biosynthesis inhibitors I Chemistry and phenomenological aspects. In: *Fungicide Resistance in Crop Protection*, eds. J Dekker & S G Georgopoulos, pp 71-86. The Pudoc, Wageningen, Netherlands.

Garthwaite D G; Thomas M R (1998). Pesticide Usage Survey Report 159: Arable farm Crops in Great Britain 1998, MAFF and SERAD. 97pp.

Hardwick N V; Slough J E; Jones D R (2000). Winter wheat: a survey of diseases. ADAS/CSL R&D. Internal report, 72pp.

Herman D; Gisi U (1994). Cross-resistance among DMI-fungicides and sensitivity distributions of *Septoria tritici* populations. *Brighton Crop Protection Conference - Pests & Diseases*, 487-492.

Kendall S J (1986). Cross-resistance of triadimenol-resistant fungal isolates to other C-14 demethylation inhibitor fungicides. *Proceedings British Crop Protection Conference - Pests & Diseases*, 539-546.

Metcalfe R J; Shaw M W; Russell P E (1998). Factors affecting strength of selection for resistance to DMI fungicides in *Septoria tritici*. *Brighton Crop Protection Conference - Pests & Diseases*, 535-540.

Pijls C F N; Shaw M W; Parker A (1994). A rapid test to evaluate *in vitro* sensitivity of *Septoria tritici* to flutriafol using a microtitre plate reader. *Plant Pathology*. **43**, 726-732.

Pijls C F N; Shaw M W (1997). Weak selection pressure by field sprays for flutriafol resistance in *Septoria tritici*. *Plant Pathology*. **46**, 247-263.

Turner J A; Elcock S J; Hims M J (1996). Monitoring triazole fungicide sensitivity in populations of *Septoria tritici*. *Brighton Crop Protection Conference - Pests & Diseases*, 695-700.

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

# Aspects of fungicide cross-resistance and implications for strobilurins

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## ABSTRACT

Trifloxystrobin is a new strobilurin fungicide developed and patented by Novartis Crop Protection AG. Due to fungicide resistance risks associated with this fungicide class, measures must be taken to protect this chemistry. One strategy is to use trifloxystrobin in programmes with fungicides from a different cross-resistance group. The cross-resistance behaviour between trifloxystrobin and a number of fungicides has been investigated. These results are discussed with regard to the validity of methods and utility of this information in the development of effective resistance management strategies.

## INTRODUCTION

Cross-resistance relationships between different fungicides are important criteria for assessing resistance risks, judging if these compounds can be used together in resistance management strategies, or if one compound can be used effectively when resistance develops against the second, and as indications of mode of action of new molecules. Cross-resistance or positive cross-resistance between two fungicides is defined as resistance to both compounds that is conferred by the same gene allele, and distinguished from multiple or combined resistance where resistance in an individual to two fungicides is due to the combined occurrence in that individual of genes which separately confer resistance to each of the fungicides (Dekker, 1987). Negative cross-resistance occurs when the same allele that confers resistance to one fungicide confers sensitivity to the second.

Proof of cross-resistance requires the crossing of resistant and sensitive parents and examination of progeny for identical segregation pattern to the two fungicides. In practice, however the determination of cross-resistance is usually achieved by testing a number of resistant and sensitive individuals to one fungicide for sensitivity to the second fungicide. If individuals from different sources consistently show the same reaction to both fungicides then positive cross-resistance is indicated. On the other hand, if individuals consistently show an opposite reaction to the second fungicide, then negative cross-resistance is indicated. If no consistent relationship can be demonstrated then there is no cross-resistance between the two compounds. Because resistance is often a quantitative character, correlation analysis is needed to clarify the relationship.

Selection for resistance however does not occur in isolation. For example, selection for aggressiveness can also result in individuals which may survive better against a range of fungicides differing in their mode of action. Since these traits may be non-specific in nature, tests with individuals which do not carry specific resistance genes may give the impression of cross-resistance at a low level between these fungicides.

### Key requirements for cross-resistance analysis

Cross-resistance analyses must include:

- a sufficient number of isolates from diverse sources for correlation analyses. This requirement improves the probability of differentiating cases of combined resistance from cross-resistance
- resistant and sensitive isolates (i.e. outside and within baseline range of sensitivity) to at least one, preferably both of the two fungicides in question

Correlation coefficients should be of a high level (usually in excess of 0.7) and must be statistically significant. Our ability to detect small degrees of correlation increases with increasing mass of experimental data. Therefore given sufficient data points, it is possible to detect small amounts of explained variance to a significant degree. These small changes in adaptation do not necessarily result in resistance and do not reflect cross-resistance between fungicide groups.

The present study reports on studies of cross-resistance between the stobilurins and other fungicide groups commonly used for the control of each of the diseases, powdery mildew on wheat, black sigatoka on banana and scab on apple. An exception was the study of cross-relationships between the strobilurins and famoxadone or fenamidone, where *Mycosphaerella fijiensis* was used as a model organism for this purpose.

## MATERIALS AND METHODS

### *Erysiphe graminis* f.sp. *tritici*

Dose response tests were conducted on detached leaf segments of wheat (cv. Kanzler) maintained on benzimidazole agar (Felsenstein 1994; Chin *et al*, 2000). The segments were treated by dipping into aqueous suspensions of a range of doses of each fungicide (trifloxystrobin 50WG, kresoxim-methyl 50WG, quinoxyfen 500SC, fenpropimorph 750EC, fenpropidin 750EC, cyprodinil 75WG and propiconazole 250EC) for 30 minutes before inoculation with single colony isolates of the pathogen obtained from diverse regions in Europe. $EC_{50}$s were calculated by probit analyses of the percentage sporulating area on each leaf segment after 10 days incubation at 20°C.

### *Mycosphaerella fijiensis*

Single ascospore isolates (4 resistant and 2 sensitive to trifloxystrobin) were tested for their sensitivity to trifloxystrobin (50WG), azoxystrobin (250SC), famoxadone (50WG), fenamidone (250SC) and propiconazole (250EC) and tridemorph (750EC), using a mycelial growth test in 24-well microtitre plates. The plates were filled with potato dextrose agar (1.5 ml per well) containing the doses 0, 0.0001, 0.01, 0.1, 1 and 10 ppm of each fungicide, inoculated with mycelial suspensions of the isolates and incubated at 24°C in the dark. The percentage growth of mycelium in the plates were assessed visually after 14 days incubation and $EC_{50}$ values were determined as described above.

### *Venturia inaequalis*

Conidial suspension aliquots (100 µl) containing 100,000-150,000 spores per ml were streaked homogeneously on fungicide amended water-agar plates (containing 200 ppm streptomycin sulfate) with a sterile spatula and incubated for 48 hours in the dark at 21°C. After incubation the growth of the germ tubes was stopped by spraying the plates with 1 % Javell solution. Germination of 150 spores (trifloxystrobin and kresoxim-methyl) or germ tube length (cyprodinil, Kueng *et al.*, 1999) at each concentration was visually assessed using a microscope and $EC_{50}$ values were determined. In the case of difenoconazole (250EC) an *in planta* test was performed. Two-week old apple seedlings were treated preventively in the dose range 0 to 100 ppm with the fungicide and inoculated with a spore suspension of the fungus. The percent sporulating leaf surface was determined after 14 days incubation and $EC_{50}$ values determined.

## RESULTS AND DISCUSSION

### *Erysiphe graminis*

There was a high level of cross-resistance between trifloxystrobin and kresoxim-methyl, but no cross-resistance was detected between trifloxystrobin and quinoxyfen, fenpropimorph, fenpropidin, cyprodinil and propiconazole (Table 1). This lack of cross-resistance was reflected in the continued high level of control of powdery mildew with fenpropidin and cyprodinil in areas of Germany with high levels of resistance to stobilurins in 1998-9 (Chin, *et al.*, 2000).

Although $r$ was significant at $p<0.002$ and 0.01 respectively for the relationships between trifloxystrobin and fenpropimorph, and between trifloxystrobin and cyprodinil, the amount of explained variance (<10%) was too low to be of biological significance.

### *Mycosphaerella fijiensis*

Cross-resistance of trifloxystrobin to azoxystrobin, famoxadone, fenamidone, but not between trifloxystrobin and propiconazole or tridemorph (Table 2) was observed. Correlation coefficients were in each case in excess of 0.99 at $p<0.001$ for the relationships between trifloxystrobin and the other respiration inhibitors, but were not significant at $p<0.05$ between trifloxystrobin and the sterol biosynthesis inhibitors, propiconazole and tridemorph.

The $EC_{50}$ values of the sensitive isolates also indicated a higher inherent activity of trifloxystrobin compared to azoxystrobin, famoxadone and fenamidone in the ascospore germination test.

Table1. Correlation analysis of log transformed $EC_{50}$ values between trifloxystrobin and kresoxim-methyl, quinoxyfen, fenpropimorph, fenpropidin, cyprodinil and propiconazole on *E. graminis.*

| Analysis | kresoxim-methyl | quinoxyfen | fenpropi-morph | fenpropidin | cyprodinil | propiconazole |
|---|---|---|---|---|---|---|
| No of isolates | 8 | 8 | 120 | 120 | 110 | 80 |
| *r* | 0.99 | 0.25 | 0.28 | 0.14 | 0.24 | 0.03 |
| *p* | 0.002 | ns | 0.002 | ns | 0.01 | ns |

ns: not significant at $P<0.05$

Table 2. Cross-resistance relationships between trifloxystrobin and azoxystrobin, famoxadone, fenamidone, propiconazole and tridemorph on *M. fijiensis.*

| | $EC_{50}$ Values (ppm) | | | | | |
|---|---|---|---|---|---|---|
| Isolate | trifloxystrobin | azoxystrobin | famoxadone | fenamidone | propiconazole | tridemorph |
| MF 97.4 | >10 | >10 | >10 | >10 | 0.3 | 1.8 |
| MF 97.8 | >10 | >10 | >10 | >10 | 0.22 | >10 |
| MF 99.10 | >10 | >10 | >10 | >10 | 1.7 | >10 |
| MF 99.17 | >10 | >10 | >10 | >10 | 6.8 | >10 |
| MF 97.25 | 0.0067 | 0.058 | 0.07 | 0.5 | 0.14 | |
| MF 97.30 | 0.0019 | 0.027 | 0.03 | 0.3 | 0.17 | 0.45 |

Table 2 (contd). Correlation analysis of log transformed values between trifloxystrobin and other fungicides.

| | azoxystrobin | famoxadone | fenamidone | propiconazole | tridemorph |
|---|---|---|---|---|---|
| *r* | 0.999 | 0.999 | 0.999 | | |
| *P* | <0.001 | <0.001 | <0.001 | ns | ns |

ns: not significant at $p<0.05$

A recent report (Jordan *et al.*, 1999) indicated that although famoxadone is not a strobilurin, it inhibits mitochondrial electron transfer at the Qo site of cytochrome bc1 as do the strobilurins. However, the authors also suggested that famoxadone shows a different binding site from that of strobilurins. It would appear from the present findings that regardless of binding behaviour, there is clear cross-resistance between strobilurins and famoxadone.

***Venturia inaequalis***

There was a high level of cross-resistance between trifloxystrobin and kresoxim-methyl, but no cross-resistance was detected between trifloxystrobin and cyprodinil and difenoconazole (Table 3).

Table 3. Correlation analysis of log transformed $EC_{50}$ values between trifloxystrobin and kresoxim-methyl, cyprodinil and difenoconazole on *Venturia inaequalis*.

| Analysis | kresoxim-methyl | cyprodinil | difenoconazole |
|---|---|---|---|
| No of isolates | 15 | 13 | 11 |
| *r* | 0.99 | 0.12 | 0.44 |
| *p* | <0.001 | ns | ns |

ns: not significant at $P<0.05$

The lack of cross-resistance between the trifloxystrobin and cyprodinil, difenoconazole, propiconazole, fenpropimorph, fenpropidin, tridemorph and quinoxyfen in the above pathogens suggests the potential of using the latter fungicides as part of anti-resistance strategies for the sustainable use of strobilurin fungicides. On the other hand the above evidence indicates that strobilurins should not be used in combination with famoxadone or fenamidone for the purposes of resistance management.

## REFERENCES


Chin, KM; Chavaillaz, DC; Kaesbohrer, M; Staub, T; Felsenstein, FG (2000). Characterizing resistance risk of *Erysiphe graminis* f.sp. *tritici* to strobilurins. *Crop Protection* (in print).

Dekker, J. (1987). Development of resistance to modern fungicides and strategies for its avoidance. In: *Modern Selective Fungicides*, ed. H Lyr, pp 23-28. Gustav Fischer: New York.

Felsenstein, FG (1994). Sensitivity of *Erysiphe graminis* f.sp. *tritici* to demethylation inhibiting fungicides in Europe. In: *Fungicide Resistance*, BCPC Monograph 60, eds S Heaney, D Slawson, DW Hollomon, M Smith, PE Russell, DW Perry, pp 35-42: Farnham

Jordan, DB; Livingston, RS; Bisaha, J; Duncan, KE; Pember, SO; Picolleli, MA; Schwartz, RS; Sternberg, JA; Tang, X-S (1999). Mode of action of famoxadone. *Pesticide Science* **55**, 105-111

Kueng, R; Chin, KM; Gisi, U (1999). Sensitivity of *Venturia inaequalis* to cyprodinil. In: Modern fungicides and antifungal compounds II, eds H Lyr, PE Russell, HW Dehne, HD Sisler, pp 313-322. Intercept: Andover.

# The latest status of resistance to strobilurin type action fungicides in Japan

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## ABSTRACT

Recently, strobilurin-resistant strains of *Sphaerotheca fuliginea* (powdery mildew) and *Pseudoperonospora cubensis* (downy mildew) were detected from many cucumber fields in Japan. These isolates showed high levels of resistance. Competitive potentials of these isolates were determined using various bioassay methods. No differences were found between sensitive and resistant isolates in the proliferation properties including their sporulation ability. When subcultured for several generations, the proportion of resistant isolates of *S.fuliginea* was shown to be stable in mixed populations of resistant and sensitive isolates. In contrast, the proportion of resistant strains of *P.cubensis* in mixed populations sometimes decreased. In the greenhouse where strobilurin-resistant strains of powdery and downy mildew had been detected, resistant strains were also found in the following cultivation.

## INTRODUCTION

In Japan, strobilurin fungicides, kresoxim-methyl and azoxystrobin, were registered for commercial use in December 1997 and April 1998 respectively. These strobilurin type action fungicides are extensively and frequently used for disease management, because of their high level of efficacy against many plant diseases. However, soon after the commercialization, there were frequent reports of poor field performance against cucumber powdery and downy mildews (Fujita *et al.*, 1999; Ishii *et al.*, 1999; Ogasawara *et al.*, 1999; Takeda *et al.*, 1999).

This paper describes the result of studies on the competitive potentials of the strobilurin-resistant isolates against sensitive ones and discusses resistance management strategies.

## DIFFERENCE OF PATHOGENICITY BETWEEN ISOLATES

For powdery mildew, the germination rate and numbers of conidia were examined, and numbers of conidia and pathogenicity of the isolates were examined for downy mildew. The results are shown in Table 1 (for *S.fuliginea*) and in Table 2 (for *P.cubensis*). Results of these bioassays showed no differences between the sensitive and resistant isolates, which indicated that sensitive and resistant isolates have similar competitive potential.

## THE CHANGE IN SENSITIVITY AFTER SUBCULTURING OF MIXTURES OF RESISTANT AND SENSITIVE ISOLATES

Various ratios of resistant and sensitive isolates were subcultured and their sensitivity

Table 1 Pathogenicity parameters of different isolates of *S.fuliginea*

| Isolate | Resistance Status | ermination rate of conidia (%) | Numbers of conidia* |
|---|---|---|---|
| IS2-1 | Resistant | 63 | 3,900 |
| IS5-1 | Resistant | 67 | 4,100 |
| SB3-1 | Resistant | 67 | 3,100 |
| M-3 | Resistant | 65 | 4,100 |
| C6-1 | Resistant | 60 | 2,600 |
| K-7-2 | Sensitive | 68 | 2,900 |
| S-2-31 | Sensitive | 69 | 2,600 |
| N-2-61 | Sensitive | 66 | 3,100 |
| W-2 | Sensitive | 68 | 2,900 |
| IS-S | Sensitive | 68 | 3,400 |

* Numbers of conidia per a leaf disk which has a diameter of 9 mm ten days after inoculation

Table 2 Pathogenicity parameters of different isolates of *P.cubensis*

| Isolate | Resistance Status | Forming rate of lesion (%) | | | Numbers of conidia** |
|---|---|---|---|---|---|
| | | $2\times10^4$ conidia/ml* | $2\times10^3$ | $2\times10^2$ | |
| 991007-12 | Resistant | 100 | 75 | 8 | 5,100 |
| 991997-13 | Resistant | 100 | 63 | 0 | 5,200 |
| 991023-1 | Resistant | 100 | 50 | 0 | 6,200 |
| Noukanken-1 | Resistant | 100 | 38 | 0 | 6,000 |
| Noukanken-2 | Resistant | 100 | 50 | 8 | 6,500 |
| MAZ-1 | Sensitive | 100 | 38 | 0 | 4,900 |
| MCZ-4 | Sensitive | 100 | 50 | 0 | 5,200 |
| FS-1 | Sensitive | 100 | 43 | 8 | 5,900 |

* Concentration of conidia in inoculation (30 $\mu$ l / one spot)

** Numbers of conidia per eight disease spots seven days after inoculation

was determined. For *S.fuliginea*, two combinations of the resistant and sensitive isolates (C6-1 and IS2-1 / N-2-61 and NG91-4) were subcultured for five generations and their sensitivity was determined using an *in vitro* assay in which leaf disks were floated in azoxystrobin solutions at concentrations of 0, 0.001, 0.002, 0.005, 0.01, 0.02, 0.2, 1, 2, 10 or 20 mg/litre 24 hours before inoculation. For *P.cubensis*, two mixtures (MA and MC / Noukanken and MC) were also studied to determine the change of populations of resistance by measuring numbers of germinating zoosporangia in azoxystrobin solution at concentration of 5mg/litre. Results are shown in Table 3 (for *S.fuliginea*), and in Figure 1 (for *P.cubensis*). For *S.fuliginea*, there were no changes in sensitivity of resistant isolates C6-1 and N-2-61. For *P.cubensis*, the proportion in the mixture of one isolate (MA) gradually decreased over successive generations whereas the proportion of the other isolate (Noukanken) did not decrease (Figure 1).

Table 3 Change in sensitivity after subculturing of mixtures of the resistant and sensitive isolates (powdery mildew)

| Azoxystrobin concentration (ppm) | Ratio of resistant isolate (C6-1) to sensitive isolate (N-2-61) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 100.0 | 99.9 | 99.0 | 90.0 | 50.0 | 10.0 | 1.0 | 0.1 | 0 |
| | Diseased degree in first generation (%)* | | | | | | | | |
| 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | 7 | 13 | 13 | 13 | 13 | 7 | 20 | 0 | 0 |
| 5 | 40 | 40 | 47 | 53 | 40 | 40 | 27 | 0 | 0 |
| 2 | 100 | 100 | 100 | 100 | 100 | 80 | 40 | 0 | 0 |
| 1 | 100 | 100 | 100 | 100 | 100 | 80 | 40 | 0 | 0 |
| 0.2 | 100 | 100 | 100 | 100 | 100 | 80 | 40 | 0 | 0 |
| 0.02 | 100 | 100 | 100 | 100 | 100 | 80 | 40 | 0 | 0 |
| 0.01 | 100 | 100 | 100 | 100 | 100 | 80 | 40 | 0 | 0 |
| 0.005 | 100 | 100 | 100 | 100 | 100 | 87 | 40 | 20 | 20 |
| 0.002 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 0.001 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 0 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| | Diseased degree in fifth generation (%)* | | | | | | | | |
| 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | 27 | 20 | 20 | 20 | 27 | 20 | 7 | 0 | 0 |
| 5 | 53 | 53 | 60 | 40 | 60 | 40 | 20 | 0 | 0 |
| 2 | 100 | 100 | 100 | 100 | 100 | 73 | 40 | 0 | 0 |
| 1 | 100 | 100 | 100 | 100 | 100 | 80 | 47 | 0 | 0 |
| 0.2 | 100 | 100 | 100 | 100 | 100 | 80 | 53 | 0 | 0 |
| 0.02 | 100 | 100 | 100 | 100 | 100 | 80 | 47 | 0 | 0 |
| 0.01 | 100 | 100 | 100 | 100 | 100 | 80 | 53 | 0 | 0 |
| 0.005 | 100 | 100 | 100 | 100 | 100 | 87 | 53 | 20 | 27 |
| 0.002 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 80 | 87 |
| 0.001 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 0 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

* Diseased degree were measured with the following disease indexes :
index 0 , percentage of lesion area = 0 ; index 1 , 1-5% ; index 2 , 6-25% ; index 3 , 26-50% ; index 4 , 51-75% and index 5 , 76-100%.
diseased degree = $\Sigma$ ( index × number of applicable disks ) × 100 / 5

## BEHAVIOUR OF RESISTANT ISOLATES IN FOLLOWING CROP

Tests were conducted to determine whether the resistant isolates could be detected in the following cultivation in the greenhouse where resistance to powdery and downy mildew had been detected in the previous cultivation.

### Survey of powdery mildew

Isolates were collected in May and in October (i.e. in the following crop) 1999 respectively in the greenhouse where failure of efficacy against powdery mildew by strobilurin type action fungicides had been reported. Some isolates were also collected in another nearby greenhouse in October. The minimum inhibition concentration (MIC) and 50% effective concentration ($EC_{50}$) values to azoxystrobin and kresoxim-methyl of these isolates were determined with using an *in vitro* assay leaf disks were floated in azoxystrobin

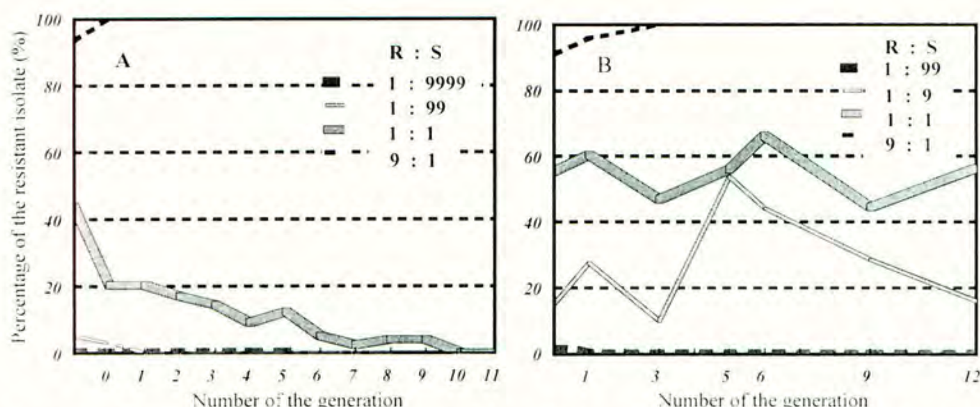


Figure 1 Change in sensitivity of mixtures of the resistant and sensitive isolates of *P.cubensis* : (A) MA/MC mixture and (B) Noukanken/MC mixture.

solutions at concentrations of 0, 0.001, 0.002, 0.005, 0.1, 0.02, 0.2, 1, 2, 10 or 20 mg/litre 24 hours before inoculation. The results are shown in Table 4. All isolates collected in the next cultivation showed high levels of resistance.

Table 4 Behaviour of the resistant isolates of *S.fuliginea* in the following crop

| Date of sampling | Isolate | Place of sampling | Sensitivity to azoxystrobin | | kresoxim-methyl |
|---|---|---|---|---|---|
| | | | MIC (ppm) | $EC_{50}$ (ppm) | MIC (ppm) |
| May, 1999 | MA | Greenhouse No.4* | 20 | 5.7 | >20 |
| May, 1999 | MC | Greenhouse No.4 | 20 | 5.7 | >20 |
| Oct, 1999 | 4-1 | Greenhouse No.4 | 20 | 6.7 | >20 |
| Oct, 1999 | 4-2 | Greenhouse No.4 | 20 | 7.7 | >20 |
| Oct, 1999 | 7-1 | Greenhouse near No.4 | 20 | 6.5 | >20 |
| Oct, 1999 | 7-4 | Greenhouse near No.4 | 20 | 6.9 | >20 |
| | K-7-2 | (Stored isolate; sensitive) | 0.01 | 0.0042 | 0.5 |
| | IS2-1 | (Stored isolate; resistant) | 20 | 9.8 | >20 |

* Greenhouse where the resistant isolates were detected in May 1999

**Survey of downy mildew**

Some isolates were collected in June and in October to November (i.e. in the following crop) 1999 respectively in the greenhouse where a few resistant strains had been detected. In another nearby greenhouse, some isolates were collected in October and then in December after azoxystrobin had been applied once. The sensitivities (MIC and $EC_{50}$) to azoxystrobin of these isolates were determined using *in vitro* leaf disk assay as described earlier. The ratios of resistant isolates to sensitive ones were also determined by measuring numbers of germinating zoosporangia in azoxystrobin solutions at concentration of 5mg/litre. The results are shown in Table 5. Six of seven isolates collected in October to November produced the resistant conidia, although the ratios of them were very low (from 0.03 to 0.2%). After azoxystrobin was applied once, the ratio of resistant isolates increased rapidly.

Table 5 Behaviour of the resistant isolates of *P.cubensis* in the following crop

| Date of sampling | Isolate | Sensitivity to azoxystrobin | | Percentage of resistant isolate |
|---|---|---|---|---|
| | | MIC (ppm) | $EC_{50}$ (ppm) | (%) |
| Greenhouse No.4* | | | | |
| Jun. 1999 | MA | >30 | - | 100 |
| Jun. 1999 | MC | 0.1 | 0.025 | 0 |
| Oct. 1999 | 1-4-2 | - | (1.4)** | 0.2 |
| Nov. 1999 | 2-4-2 | - | (3.2) | 0.2 |
| Nov. 1999 | 2-4-3 | - | (3.0) | 0.1 |
| Nov. 1999 | 2-4-4 | - | (3.1) | 0.4 |
| Nov. 1999 | 2-4-5 | - | (2.3) | 0.04 |
| Greenhouse near No.4 | | | | |
| Oct. 1999 | 1-7-1 | 0.2 | 0.076 | 0 |
| Oct. 1999 | 1-7-2 | 0.5 | 0.076 | 0.03 |
| *Azoxystrobin had been applied once before the isolates were collected in Dec.* | | | | |
| Dec. 1999 | 3-7-1 | >30 | - | 99 |
| Dec. 1999 | 3-7-2 | >30 | - | 48 |
| Dec. 1999 | 3-7-3 | >30 | - | 47 |
| Dec. 1999 | 3-7-4 | >30 | - | 96 |
| Dec. 1999 | 3-7-5 | >30 | - | 43 |

* Greenhouse where the resistant isolates were detected in June 1999

** Reference value : exact values could not be measured with this method (Uchida *et al*., 2000

## DISCUSSION

Development of resistance is a critical factor which can affect fungicide efficacy. It is very important to determine the competitive potentials of the resistant isolates and to study other control methods for resistance management in fields where resistant ones appear. If the competitive potential of a resistant isolate was lower than that of a sensitive one, the ratio of resistant isolates might decrease and it could allow the same chemical to be used again. In practice, there are some resistance management strategies that interrupt the application of the chemicals concerned temporarily or which alternate their use with chemicals from different cross-resistance groups. For such strategies to be successful, resistant isolates must have a lower competitive potential than sensitive isolates.

However, in these experiments, it was shown that there were no differences in competitive potential between the sensitive and resistant isolates of *S.fuliginea* and of *P.cubensis*. Moreover, in the greenhouse where the resistant isolates of *S.fuliginea* and *P.cubensis* had been detected, resistant isolates were detected in the following crop. In addition, the ratio of the resistant isolates of *P.cubensis* increased rapidly after one application of azoxystrobin. Furthermore, Fuji *et al.*, (2000) have shown that the ratio of resistant isolates remained constant when the application of strobilurin type action fungicides was ceased.

The strobilurin-resisitant isolates of *S.fuliginea* and *P.cubensis* detected in Japan show high levels of resistance. The appearance of resistant strains is very likely to result in poor disease control and crop loss. Therefore, these results suggest us that we must make efforts to prevent the occurrence of resistance to strobilurin type action fungicides.

The strobilurin type action fungicides are very useful for disease control and crop supply, not only because they have a high level of efficacy against many plant diseases but also because they are broken down rapidly in the environment. Therefore, it is very important that consideration is given to their pattern of use in order to prevent or delay the development of resistance. Based on these studies, we propose that the number of applications of strobilurin type action fungicides within one cultivation period must be limited to one and that their use is alternated with effective fungicides from different cross-resistance groups within a total disease management programme.

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## REFERENCES

Fuji M; Kawagoe Y; Uchida K; Takeda T; Ishii H; Amano T (2000). Biological properties of strobilurin-resistant isolates of *Pseudoperonospora cubensis*. *Annals of the Phytopathological Society of Japan*, in press.

Fujita N; Hamada K; Inagaki M, Enoyoshi T; Miyahara T; Kojiguchi S; Ogasawara K; Kadota G; Dale S M; Takamatsu S (1999). Strobilurin baseline determination and resistance monitoring. *Annals of the Phytopathological Society of Japan*, **65**, 692.

Ishii H; Noguchi K; Tomita Y; Umemoto S; Nishimura K (1999). Occurrence of strobilurin resistance in powdery mildew and downy mildew on cucumber. *Annals of the Phytopathological Society of Japan*, **65**, 655.

Ogasawara K; Enoyoshi T; Miyahara T; Kojiguchi S; Kadota G; Takamatsu S; Dale S M (1999). Cucurbit powdery mildew resistant to Strobilurins. *Annals of the Phytopathological Society of Japan*, **65**, 655.

Takeda T; Kawagoe Y; Uchida K; Fuji M; Amano T (1999). The appearance of resistant isolates to strobilurins. *Annals of the Phytopathological Society of Japan*, **65**, 655.

Uchida K; Kawagoe Y; Fuji M; Takeda T; Amano T (2000). Testing methods for sensitivity of *Pseudoperonospora cubensis* to new fungicides and baseline sensitivity. *Annals of the Phytopathological Society of Japan*, in press.

# Sensitivity of powdery mildew and yellow rust to DMI, morpholine and strobilurin fungicides in England and Scotland

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## ABSTRACT

Recent UK studies of the resistance status of wheat and barley mildew and wheat yellow rust to DMI, morpholine and strobilurin fungicides are described. The sensitivity of both wheat and barley mildew to DMIs and morpholines has shifted significantly towards resistance compared with baseline sensitive isolates. The shift has been greater for DMIs than for morpholines and for wheat mildew than barley mildew. A small number of strobilurin-resistant isolates of wheat mildew were detected in 1999, but, as yet, resistance has not been detected in barley mildew. There was evidence of a shift towards insensitivity to DMI fungicides in wheat yellow rust during the late 1990s.

## INTRODUCTION

UK farmers are heavily dependent on fungicides for disease control in cereal crops. This means that any reduction in the efficacy of a fungicide due to reduced sensitivity can have serious economic consequences.

Although wheat and barley cultivars with a high degree of genetic resistance to mildew (*Erysiphe graminis*) are available to growers, fungicides still play an important role in controlling the disease in susceptible cultivars. Similarly, for yellow rust of wheat, the popularity of certain highly susceptible cultivars in recent years has contributed to epidemics and increased requirements for fungicidal control.

Morpholines and DMIs (sterol demethylation inhibitors – triazoles) have been the standards for mildew control over the past 30 years. DMIs were initially used intensively to control mildew and by the mid 1980s their performance had declined to a level at which disease control failure was commonly encountered in the field (Clark, 1992). Insensitivity to the first morpholine fungicide, ethirimol, was reported in 1979 (Walmsley-Woodward *et al*., 1979) and a shift towards insensitivity to fenpropimorph, introduced in the early 1970s, was detected in 1986 (Wolfe *et al*., 1987). Despite this, morpholines still give generally good control of mildew in the field. Control of yellow rust of wheat (*Puccinia striiformis*) on susceptible varieties is heavily dependent upon DMI fungicides, with morpholines sometimes being used for their quick eradicant effect. An investigation of yellow rust isolates collected between 1960 and 1990 gave no

evidence of any changes in sensitivity to DMIs or morpholines over the period (Barnard, 1992; Bayles *et al.*, 1992; Bayles *et al.*, 1994).

The introduction of the strobilurin fungicides, effective against a very wide spectrum of pathogens, has created new possibilities for the control of mildews and rusts, as well as other important diseases. However, DMIs and morpholines are likely to continue as vital components of fungicide programmes, both for efficacy reasons and for considerations of resistance management. Strobilurins have a unique mode of action involving the target pathogen's mitochondrial adenosine triphosphate (ATP) production. It was thought initially that this would be a difficult mechanism to corrupt and that the risk of resistance would therefore be low. However, strobilurin-resistant wheat mildew isolates were found in Germany in 1998 (FRAC, 1998) and more widely throughout Europe in 1999. Resistance to strobilurins in barley mildew was reported in Germany in 1999. There do not appear to be any studies of the sensitivity of yellow rust to strobilurins. Strobilurin resistance in wheat mildew arose in a single step, with full rates of the fungicide being totally ineffective against resistant isolates. This implies a single gene resistance.

Regular and independent monitoring of fungicide sensitivity in pathogen populations is essential to prevent unexpected and widespread loss of efficacy of key chemical groups. For new chemical groups, monitoring establishes a baseline sensitivity, against which future changes can be measured. Monitoring also provides essential information for evaluating anti-resistance strategies and for estimating the risk of resistance developing in a particular chemical group.

This paper presents an update on the resistance status, in the UK, of wheat and barley mildew and wheat yellow rust to three major groups of fungicides – DMIs, morpholines and strobilurins. The results are drawn from a number of studies carried out by NIAB and SAC over the past four years.

## MATERIALS AND METHODS

### Mildew

Wheat and barley mildew samples were taken from fungicide trials at 13 sites in the UK in 1998 and at 15 sites in 1999. Samples were collected from plots receiving a range of different fungicide programmes, including conventional chemistry alone and various combinations of conventional and strobilurin chemistry. In 1998, all samples were collected after the final spray of each programme. In 1999, additional samples were collected before the start of spraying and, in some trials, part way through the spray programme.

Mildew isolates were maintained in the laboratory on universally susceptible cultivars of wheat and barley, either using seedlings growing in isolation propagators or detached leaf segments on agar in plastic boxes.

A total of 450 isolates of wheat mildew and 245 isolates of barley mildew were tested for fungicide sensitivity using detached leaf assays. Isolates were tested using established

methods for sensitivity monitoring studies (Burnett, 1999; S.Slater, pers. comm.). Seedlings of the susceptible cultivar were sprayed with a range of fungicide concentrations and an untreated control was sprayed with water. Leaf segments cut from the sprayed seedlings were placed on agar containing 80-100 ppm benzimidazole and inoculated evenly with spores of each test isolate. At least two sensitive control isolates were included in each test. After approximately 14 days incubation, the surface area of the leaf segments infected by mildew was assessed. From this, $EC_{50}$ values were estimated for each isolate. Resistance factors (RF) were calculated as the $EC_{50}$ of a test isolate divided by the mean $EC_{50}$ of the sensitive control isolates. In 1998, isolates were tested for sensitivity to DMIs (using epoxiconazole or tebuconazole), morpholines (using fenpropimorph) and strobilurins (using azoxystrobin). In 1999, isolates were tested for sensitivity to strobilurins only.

**Yellow rust**

60 isolates of wheat yellow rust collected by the UK Cereal Pathogen Virulence Survey during 1997 and 1998 were tested for sensitivity to the DMI fungicide triadimenol. Two reference isolates, used in an earlier survey of fungicide insensitivity in yellow rust during the period 1960-1990 (Barnard, 1992), were included in all tests to allow comparisons to be made with the previous study. One of these represented median sensitivity prior to 1991 and the other represented the insensitive extreme of the range during the same period. A seedling test method, described by Bayles (1998) was used. $EC_{50}$ values were estimated for each isolate and compared with those of the reference isolates.

Further samples of wheat yellow rust were collected from fungicide trals in 1998 and 1999. 33 isolates were tested for sensitivity to the strobilurin fungicide azoxystrobin, using a seedling test method based on that of Barnard (1992). $EC_{50}$ values were estimated and a resistance factor calculated for each isolate compared with two control isolates, collected in surveys before the deployment of strobilurin fungicides.

**RESULTS**

Figure 1 shows the distribution of resistance factors (RF) for the sensitivity of barley mildew isolates to DMI and morpholine fungicides in 1998 and to strobiliurins in 1998 and 1999. An RF value of 1.0 represents the sensitivity of the sensitive baseline control isolates. The distribution of RFs for DMIs was further towards insensitivity than that for morpholines, with nearly 70 percent of isolates having RF values greater than 16 for DMIs compared with around 15 percent for morphlines. RF values for sensitivity to strobilurins occupied a narrow range on either side of the control isolates, with maximum values in the 4.0-7.9 group. Distributions in 1998 and 1999 were almost identical.

Figure 2 shows RF distributions for the sensitivity of wheat mildew isolates to DMI and morpholine fungicides in 1998 and to strobilurins in 1998 and 1999. It also shows the distribution of RF values for the sensitivity of yellow rust isolates to strobiliurins, for the two years together. As with barley mildew, the distribution of RFs for DMIs was further towards insensitivity than the distribution for morpholines. For strobilurin sensitivity

there was a narrow distribution of low RF values in 1998, similar to that found in barley mildew. However, in 1999, three strobilurin-resistant isolates were detected, resulting in a discontinuous distribution of RFs. These isolates were fully resistant, showing very little reduction in growth at rates of azoxystrobin equivalent to twice the full dose. It was therefore impossible to calculate true $EC_{50}$s or RFs. The distribution of RF values for the sensitivity of wheat yellow rust isolates to strobilurins was similar to that for wheat mildew in 1998 and barley mildew in both years.

Figure 3 compares the sensitivity of wheat yellow rust isolates to the DMI fungicide triadimenol in 1997 and 1998 with the sensitivity of isolates from the 1960-1990 period, tested as part of an earlier project (Barnard, 1992). $EC_{50}$ and RF values could not be calculated for the 1960-1990 data as tests had been designed to compare the performance of test and reference isolates at a single fungicide dose only. Equally, the reference isolates had been selected to represent median and high insensitivity in the 1960-1990 period, rather than baseline sensitivity. Isolates have therefore been classified as 1) more sensitive than isolate WYR 83/62, 2) intermediate in sensitivity between isolates WYR 83/62 and WYR 90/20 and 3) more insensitive than isolate WYR 90/20. The results show that, during the years 1960-1990, slightly more than half the isolates tested were more sensitive than WYR 83/62 and only 2% were less sensitive than WYR 90/20. In contrast, in 1997-1998, only 17% of isolates were more sensitive than WYR 83/62, while 54% were less sensitive than WYR 90/20. This is evidence of a significant shift in sensitivity in 1997 and 1998 compared with earlier years.

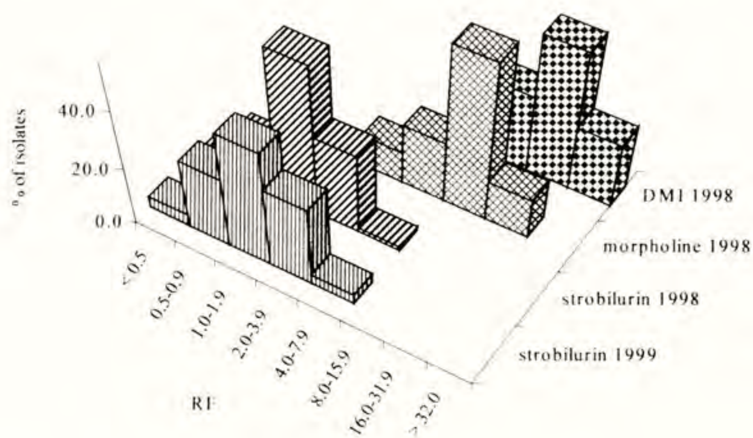


Figure 1 Distributions of RF values for barley mildew isolates

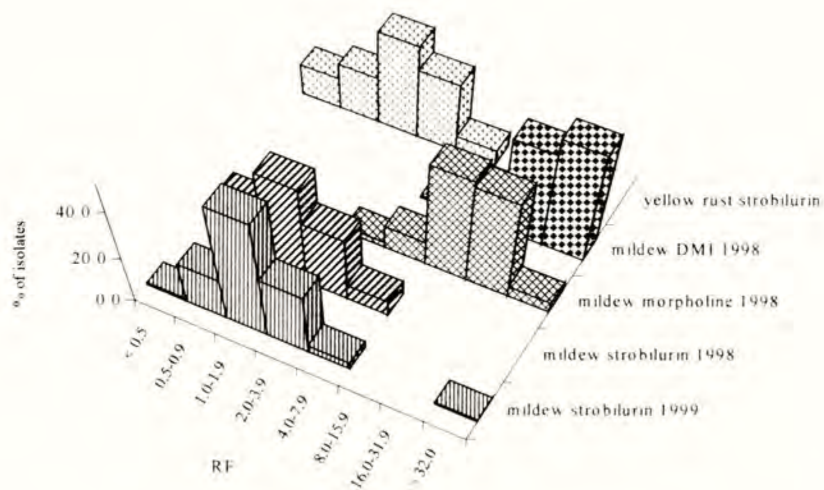


Figure 2. Distributions of RF values for wheat mildew and yellow rust isolates

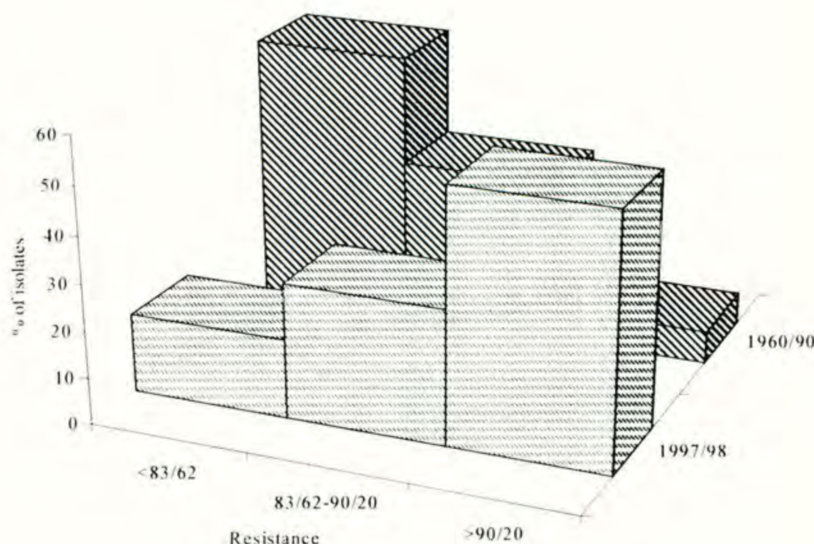


Figure 3. Resistance of wheat yellow rust isolates in 1997/1998 and 1960/1990 – relative to two reference isolates (83/62 and 90/20)

## DISCUSSION

These studies provide an update on the current resistance status of wheat mildew, barley mildew and wheat yellow rust with respect to key fungicide groups in the UK. The sensitivity of both wheat and barley mildew to DMIs and morpholines has clearly shifted since fungicides in these groups were introduced some 30 years ago. This is hardly surprising considering the continuous widespread use of this chemistry over a long period and the intrinsically high resistance risk of the pathogens concerned. The shift towards resistance has been greater for DMIs than for morpholines and for wheat mildew than for barley mildew. Average resistance factors for wheat mildew isolates in 1998/1999 were 31.8 for DMIs and 16.7 for morpholines, indicating a 32-fold or 17-fold increase in the dose of fungicide required to reduce the growth of the pathogen by 50%, when compared with unselected baseline isolates. Comparable resistance factors for barley mildew were 23.5 for DMIs and 10.7 for morpholines. Further monitoring would be needed to determine whether the sensitivity of these pathogens has stabilised or is continuing to decline. With the advent of new chemical groups with activity against mildew, there would seem to be the opportunity to stabilise the position of the older

chemistry by use of mixtures and alternations of fungicides with different modes of action.

When the strobilurin sensitivity monitoring described here started, it was widely believed that the risk of resistance to this new chemistry was low. Whether resistance, if it did develop, would do so as a gradual shift or in a single large step, was unknown.
However, in the first year of monitoring, 1998, resistance to strobilurins was reported in wheat mildew in Germany and it became clear that resistance is controlled by a major gene and occurs as a single step, resulting in two distinct populations of mildew, i.e. sensitive and resistant. The following year, 1999, this UK project detected resistance in wheat mildew in isolates collected in East Anglia. This was confirmed by monitoring results released by the agrochemical industry's Fungicide Resistance Action Committee (FRAC), a specialist group of the Global Crop Protection Federation (GCPF) dedicated to prolonging the effectiveness of fungicides liable to encounter resistance problems. Resistant isolates show little or no reduction in growth on seedlings sprayed with azoxystrobin at full dose rate. Judging by the rate at which resistance in wheat mildew appeared throughout Europe, it seems likely that the incidence of resistance in the UK will increase to high levels within one or two years, rendering strobilurins totally ineffective in controlling mildew. There is clearly a high risk that barley mildew will follow the same course.

The results of the 1997/98 survey of yellow rust insensitivity demonstrated clearly that there has been a shift towards insensitivity to DMI fungicides in the wheat yellow rust population. This shift took place during a period when the highly susceptible variety Brigadier was being grown on around 20% of the national wheat acreage. Weather conditions in 1997 and 1998 were generally favourable for yellow rust development, but often unsuitable for spraying operations at critical timings. The combination of cultivar susceptibility and weather conditions, together with a tendency for fungicides to be used at rates well below those recommended, resulted in widespread yellow rust infection and inadequate control. In response to the high disease risk, DMIs were used repeatedly in many crops, starting with seed dressings and followed by foliar applications. On theoretical grounds, prolonged exposure to a fungicide is generally believed to increase selection for insensitivity and may well have contributed to the shift observed here.

Although there have been no confirmed cases of insensitivity causing failure of yellow rust control in the field, it is clearly important that every effort should be made to halt the sensitivity shift. Appropriate anti-resistance measures include growing resistant varieties, following the principles of variety diversification and spraying at the first signs of infection using adequate dose rates. Repeated applications of DMIs alone should be avoided by using co-formulations, recommended tank-mixes or sequences of sprays which bring together different modes of action effective against yellow rust (FRAG-UK, 2000). There is also a clear risk that yellow rust may develop resistance to strobilurins and this is likely to occur as a single-step process.

## ACKNOWLEDGMENTS

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## REFERENCES

Barnard E G (1992). Fungicide insensitivity in yellow rust of wheat and barley. *Project Report No. 55*, HGCA: London.

Bayles R A (1998) Yellow rust of wheat – survey of sensitivity to DMI fungicides in 1997. *Project Report No. 175*, HGCA: London.

Bayles R A, Stigwood P L, Barnard E G (1994). Sensitivity to morpholine fungicides in yellow rust of wheat (*Puccinia striiformis*). In: *Fungicide Resistance*. BCPC Monograph No 60, 309-314.

Bayles R A, Barnard E G, Stigwood P L (1992). Fungicide sensitivity in yellow rust of wheat (*Puccinia striiformis*). *Brighton Crop Protection Conference - Pests and diseases - 1992*, **1**, 165-170.

Burnett F J (1999). Identification of effective strategies for mixing novel and conventional fungicide groups to control powdery mildew in wheat. *Project Report No. 202*, HGCA: London.

Clark W S (1992). Practical aspects of resistance to DMI fungicides in barley powdery mildew *Erysiphe graminis*. *Proceedings. Brighton Crop Protection Conference - Pests and diseases - 1992*, **3**, 177-182.

FRAC (1998). *1998 status report and recommended fungicide resistance management guidelines*. FRAC: Brussels.

FRAG-UK (2000). *Guidelines for preventing and managing fungicide resistance in cereal pathogens*. HGCA: London.

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

Wolfe M S, Slater S E, Minchin P N (1987). Mildew of barley. *UK Cereal Pathogen Virulence Survey Annual Report for 1986*, 26 - 33.

# Assessment of mefenoxam resistance in *Phytophthora erythroseptica* in the USA

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## ABSTRACT

A three-year survey was conducted from 1997-1999 to determine the sensitivity of *Phytophthora erythroseptica* to mefenoxam (metalaxyl). Previous studies had suggested that the occurrence of mefenoxam resistance was restricted to the northeastern areas of the United States, such as Maine and New York. The current survey demonstrates that the highest proportion of mefenoxam resistance is in the potato production area where it was first reported. We report here, however, the occurrence of mefenoxam resistance in *P. erythroseptica* in Idaho. The highest percentage (range 70.8-85.5%) of isolates recovered during the survey was sensitive to mefenoxam with $ED_{50}$ values ranging from 0.03-0.15 mg/litre. There were a few isolates with $ED_{50}$ ranging from 0.16-70 mg/litre. The frequency of mefenoxam resistance ($ED_{50}$ >100 mg/litre) in soil populations of *P. erythroseptica* was 79.4% in 1998 and 100% in 1999. A judicious use of mefenoxam to control pink rot in fields with *P.erythroseptica* resistant to mefenoxam is recommended.

## INTRODUCTION

Pink rot, caused by *Phytophthora erythroseptica*, is one of the most important storage diseases of potato in the United States. Infection of stolons and tubers by this pathogen can occur at anytime during the growing season when conditions favour development of the disease. Management of pink rot includes crop rotation, planting potato crops in well-drained soils, avoiding excessive irrigation at the end of the growing season, and applications of mefenoxam to the potato crop during the growing season.

Potato storage rot surveys conducted in the late-1980s and early-1990s by our research group found no evidence of mefenoxam (metalaxyl) resistance in *P. erythroseptica* (Stack *et al.*, 1993). Subsequent to our work, resistance to mefenoxam in *P. erythroseptica* was reported in potatoes grown in the northeastern portion of the US (Lambert & Salas, 1994; Goodwin & McGrath, 1995). Detection of fungicide resistance in a pathogen population is an integral component in the development of a resistance management programe. The objectives of the current work were to determine the extent of mefenoxam resistance in *P. erythroseptica* in the U.S. and develop preliminary information of the frequency of resistance in soil populations of the fungus.

## METHODS

### Isolation of *P. erythroseptica* from tubers

A total of 222, 236, and 580 tubers with symptoms of pink rot from commercial potato fields

or storages bins in the USA were received in 1997, 1998 and 1999, respectively. To isolate *P. erythroseptica*, pieces of infected tuber tissue (4x4 mm) were cut and placed on petri dishes containing water agar amended with ampicillin (100 mg/litre) and incubated in darkness at 17-20°C for 3-5 days. Fungal colonies with mycelia resembling *P. erythroseptica* were selected and purified by hyphal tipping. Occasionally the selective medium $P_5$ARPH (Jeffers & Martin, 1986) was used to isolate *P. erythroseptica* from badly rotted tubers or from tuber samples arriving late fall or winter. After isolation, *P. erythroseptica* cultures were maintained on clarified V-8 medium (100 ml V-8 juice, 1 g $CaCO_3$, 20 g Agar, 900 ml distilled water) until tested for their sensitivity to mefenoxam.

**Bioassay of *P. erythroseptica* from soil**

Soil samples (c. 0.75 kg) were collected at random or from low and wet areas of fields naturally infested with *P. erythroseptica* in Idaho (Table 1). Soil samples were stored in sealed plastic bags at room temperature (20-23°C) until bioassay within 4-7 months after their collection. Tubers of Russet Norkotah, a cultivar highly susceptible to *P. erythroseptica*, were used to trap the fungus soil samples. Tuber tissue cylinders (4 cm long x 0.7 cm diameter.) were excised from the apical and stem end of each tuber with a cork borer (No.3) to produce empty cavities. These cavities were filled with approximately 2 g of a soil sample, moistened to saturation with an autoclaved soil extract (100 g soil, 900 ml of deionized $H_2O$) when needed, and capped with an outer disc (0.5 cm) cut from the same excised tuber plug. Tubers were placed in plastic moist chambers lined top and bottom with moist paper towels and incubated at 20°C. After 10 days, tubers were cut lengthwise, through both ends, and covered with moist paper towels for 30 minutes. The number of soil cores for each soil site producing symptoms of pink rot was recorded, and isolations were made on water agar amended with ampicillin as described above. Isolates were purified by hyphal tipping and cultured on modified V-8 juice agar.

**Mefenoxam sensitivity tests**

A total of 69 (1997), 137 (1988) and 233 (1999) isolates of *P. erythroseptica* recovered from pink rotted tubers were tested to determine their sensitivity to different concentrations of metalaxyl (1997) or mefenoxam (1998-1999). Metalaxyl or mefenoxam was added to clarified V-8 agar (50 ml V-8 juice filtered through four layers of cheesecloth, 940 ml of distilled water, and 20 g agar) before autoclaving. Concentrations ranged from 0.1 to 100 a.i. mg/litre for 1997 and 1998 isolates and from 0.01 to 100 a.i. mg/litre for 1999 isolates. Culture plates with or without (control) fungicide were prepared three days before tests were performed.

Mycelial plugs, 5 mm in diameter were cut from the margin of actively growing colonies of 7-day-old cultures of *P. erythroseptica* growing on modified V-8 juice agar (100 ml V-8 filtered through four layers of cheesecloth, 900 ml distilled water, and 20 g agar). These mycelial plugs were placed in the centre of a 9-cm petri plate with the mycelium in contact with the test medium. There were two replicate culture plates for each metalaxyl or mefenoxam concentration. For each replicate a separate dilution of metalaxyl or mefenoxam was prepared. Control plates contained neither metalaxyl nor mefenoxam.

### Determination of $ED_{50}$ values

Growth of the test isolates was determined by measuring colony diameters in two perpendicular directions on each culture plate after 7 days of incubation in darkness at 20°C. Measurements were averaged and the diameter of the mycelial plug was subtracted. The relative growth reduction for each rate of mefenoxam was calculated as follows: [100-(growth with mefenoxam/growth in control plate)*100]. The concentration causing 50% ($ED_{50}$) relative reduction of mycelial growth compared to the control without mefenoxam was estimated by plotting the percent inhibition against the log-scale of mefenoxam concentration (1997, 1998) or by regression and curve fitting (1999).

## RESULTS

### Isolation of *P. erythroseptica* from tubers

A total of 435 isolates of *P. erythroseptica* were isolated from 1038 tubers with symptoms of pink rot received from 13 states of the USA and one Canadian province during a survey conducted from 1997 to 1999. The majority of the samples came from six states (Figure 1). Most (85.5%) of *P. erythroseptica* isolates collected in 1997 had $ED_{50}$ values <0.15 mg/litre, while two isolates obtained from Maine had $ED_{50}$ >100 mg/litre. Results from 1998 were similar to those from 1997 as most isolates (82.5%) had $ED_{50}$ values <0.15 mg/litre. A large proportion of *P. erythroseptica* isolates collected from Idaho fell into this range (71.7%), however, 19.6% of the isolates had $ED_{50}$ values >100 mg/litre. Most of the 1998 *P. erythroseptica* isolates from Maine had $ED_{50}$ values >100 mg/litre (61.5%) relative to those with $ED_{50}$ <0.15 (15.4%). In 1999, the proportion of isolates with $ED_{50}$ values <0.15 mg/litre decreased (70.8%) in relation to frequencies obtained in the previous two years. This trend was particularly evident in *P. erythroseptica* isolates collected from Idaho where the frequency of isolates with <0.15 (47%) or >100 mg/litre (52.9%) were similar. Interestingly, most of the isolates with $ED_{50}$ >100 mg/litre from Idaho were recovered from a single location. Maine samples had a greater proportion of *P. erythroseptica* isolates with $ED_{50}$ values >100 mg/litre (75%). Isolates of *P. erythroseptica* collected from Colorado, Minnesota, Nebraska, North Dakota, South Dakota, Texas, Washington, and Wisconsin during the years of this study had $ED_{50}$ values <0.15 mg/litre, with most isolates having $ED_{50}$ values within 0.05-0.10 mg/litre.

### Bioassay of *P. erythroseptica* from soil

In 1998, *P. erythroseptica* was recovered from all 7 fields sampled where pink rot was observed. In contrast, the pathogen was not recovered from a control field where pink rot was not previously observed (Table 1). Frequency of recovery of *P. erythroseptica* ranged from 10-30% of samples collected within fields, and from 5-65% in sub-samples from individual field samples. Five fields yielded only isolates with $ED_{50}$ values >100 mg/litre. One isolate with $ED_{50}$=0.16 mg/litre and three isolates with values >100 mg/litre were recovered from one site in Field No. 10, two sites in the same field produced isolates with $ED_{50}$ values >100 mg/litre. Three sites in wet areas of Field No. 34 were found to be infested with *P. erythroseptica*. All 13 isolates from one site had $ED_{50}$ ranging from 0.31-1.5 mg/litre, and two isolates from the other two sites had $ED_{50}$ values >100 mg/litre. All 12 isolates from three random sites of Field No. 34 had $ED_{50}$ values >100 mg/litre. Overall, of the 69 isolates

recovered from seven fields in 1998, 14 (20.3%) isolates had $ED_{50}$ values ranging from 0.16 – 1.5 mg/litre, and 55 (79.7%) isolates had $ED_{50}$ values of >100 mg/litre.

Results of soil bioassay in 1999 were similar to those of 1998. Nine sites (18% of those sampled) in Field No. 7 were found to be infested with *P. erythroseptica*. One of these sites yielded an isolate with an $ED_{50}$ value of 26 mg/litre, and seven other isolates with $ED_{50}$ values >100 mg/litre. The remaining eight sites from this field yielded only isolates with $ED_{50}$ value of >100 mg/litre. All 28 (100%) isolates recovered from seven sites (14% of those sampled) from Field No. 34 had $ED_{50}$ values of >100 mg/litre. From a total of 55 isolates of *P. erythroseptica* recovered in 1999, one isolate (1.8%) had $ED_{50}$ value of 26 mg/litre and 54 (98.2%) isolates had $ED_{50}$ value of >100 mg/litre.

Table 1. Incidence and sensitivity to mefenoxam of *Phytophthora erythroseptica* isolates from soils collected in Idaho

| Year | Field No. | No. sites sampled | No. sites infested | No. isolates recovered | No. isolates ($ED_{50}$) <26 mg/litre | >100 mg/litre |
|---|---|---|---|---|---|---|
| 1998 | 2 low area | 10 | 2 | 7 | 0 | 7 |
| | 3 low area | 10 | 1 | 1 | 0 | 1 |
| | 6 low area | 10 | 1 | 1 | 0 | 1 |
| | 10 low area | 10 | 3 | 8 | 1 (0.16) | 7 |
| | 15 low area[a] | 10 | 0 | 0 | 0 | 0 |
| | 33 low area | 10 | 2 | 15 | 0 | 15 |
| | 33 random | 10 | 3 | 8 | 0 | 8 |
| | 34 low area | 10 | 3 | 15 | 13 (0.3-1.5) | 2 |
| | 34 random | 10 | 3 | 9 | 0 | 9 |
| | 59 low area | 10 | 2 | 4 | 0 | 4 |
| 1999 | 7 | 50 | 9 | 26 | 1 (26) | 25 |
| | 32 | 50 | 7 | 28 | 0 | 28 |
| | 33 | 50 | 0 | 0 | 0 | 0 |
| | 58 | 30 | 0 | 0 | 0 | 0 |
| | 59 | 30 | 0 | 0 | 0 | 0 |

[a]Control field: Pink rot not observed

## DISCUSSION

The basis of any effective pesticide resistance management programe must include the identification and detection of insensitivity in the pest population. A 1992 survey showed that all isolates of *P. erythroseptica* from several locations in North America were sensitive to metalaxyl (Stack *et al.*, 1993). However, soon after this study, *P. erythroseptica* metalaxyl insensitive isolates were found in Maine (Lambert & Salas, 1994), and in New York (Goodwin & McGrath, 1995). Our data confirms these earlier reports that mefenoxam insensitive populations of *P. erythroseptica* are present in the northeastern states of the USA. We report here for the first time that *P. erythroseptica* populations insensitive to mefenoxam are also present in Idaho. Our data shows that the majority of *P. erythroseptica* isolates collected for this study have $ED_{50}$ values <0.15 a.i. mg/litre (Figure 1). Studies dealing with *Phytopthora parasitica* var. *nicotianae* (Csinos & Bertrand, 1994) and *Phytophthora nicotianae* (Timmer *et al.*, 1998) documented similar values.

Data on *P.erythroseptica* collected from Idaho soils shows that a high frequency of these isolates were insensitive to mefenoxam ($ED_{50}$ >100 a.i. mg/litre). However, the insensitive isolates were only from one small growing area in one county of Idaho. Work is underway to determine factors responsible for this high proportion of resistant isolates. In contrast, a large proportion of *P. erythroseptica* isolates (71.7%) collected from potato tubers in 1998 exhibiting pink rot symptoms were sensitive to mefenoxam ($ED_{50}$ < 0.15 a.i. mg/litre). In 1999, the proportion of isolates with ED values <0.15 or >100 mg/litre were similar (47% and 52.9%). These results may indicate that our survey to find insensitivity within the *P. erythroseptica* population obtained from tubers with symptoms of pink rot is skewed and does not accurately represent the populations of sensitive/insensitive isolates that exist in the soils. It also may suggest that populations of *P. erythroseptica* insensitive to mefenoxam have increased in Idaho, and a judicious use of mefenoxam is needed.

Further studies should be initiated to correlate the proportion of sensitive/insensitive isolates present in a specific site by trapping *P. erythroseptica* from soil as well as from symptomatic tubers. These studies are being performed in concert with studies evaluating the use of mefenoxam as an 'in furrow', at planting application as opposed to the prophylactic application currently used. It also may be important to continue monitoring the development of *P. erythroseptica* populations insensitive to mefenoxam to ensure proper use of this fungicide for the control of pink rot.

## ACKNOWLEDGEMENTS

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## REFERENCES

Csinos A S; Bertrand P E (1994). Distribution of *Phytopthora parasitica* var. *nicotianae* races and their sensitivity to metalaxyl in Georgia. *Plant Disease*. **78**, 471-474.

Goodwin S B; McGrath M T (1995). Insensitivity to metalaxyl among isolates of *Phytophthora erythroseptica*. *Plant Disease*. **79**, 967.

Jeffers S N; Martin S B (1986). Comparison of two media selective for *Phytophthora* and *Pythium* spp. *Plant Disease*. **70**,1038-1043.

Lambert D H; Salas B (1994). Metalaxyl insensitivity of *Phytophthora erythroseptica* isolates causing pink rot of potato in Maine. *Plant Disease*. **78**,1010.

Stack R W; Salas B; Gudmestad N C; Secor G A (1993). The lack of evidence for metalaxyl tolerance in *Phytophthora erythroseptica*, the cause of pink rot of potato. *Phytopathology* **83**, 886 (Abstract).

Timmer L W; Graham J H; Zitko S E (1998). Metalaxyl-resistant isolates of *Phytophthora nicotianae*: Occurrence, sensitivity, and competitive parasitic ability on citrus. *Plant Disease*. **82**, 254-261.

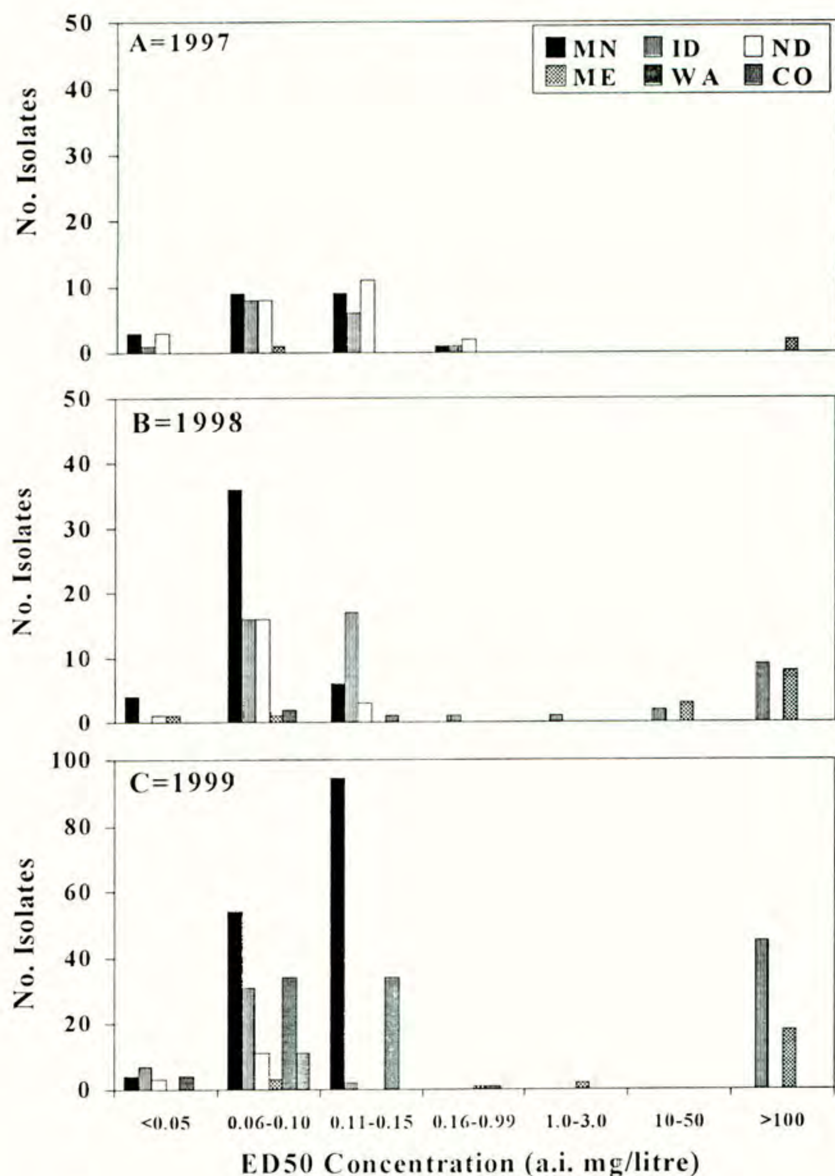


**Figure 1.** Sensitivity of *Phytophthora erythroseptica* to mefenoxam in isolates collected from throughout the USA. MN=Minnesota, ID=Idaho, ND=North Dakota, ME=Maine, WA=Washington, CO=Colorado.

# Involvement of p-glycoprotein in insecticide toxicity to *Culex pipiens* mosquitoes and implications for control programmes

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## ABSTRACT

Pesticide resistance has parallels with multidrug resistance syndrome in clinical medicine, which has been linked to an ATP-dependent pump, p-glycoprotein (P-gp). P-gps pump drugs out of the cell, thereby reducing cellular concentrations of the chemical. P-gps have been found in several invertebrate species and have been shown to provide a defence against environmental xenobiotics, including pesticides.

This study was to determine whether i) P-gps were found in *Culex pipiens* mosquitoes and ii) whether P-gps regulated pesticide toxicity. Western blotting revealed that a P-gp monoclonal antibody (C219) bound to an 85kDa protein in *C. pipiens* larvae. The P-gp modulator verapamil increased toxicity to endosulfan but not to chlorpyrifos. The implications of these results are discussed.

## INTRODUCTION

Insecticide resistance in mosquitoes is an enormous problem world-wide, with resistance to several insecticide classes simultaneously becoming commonplace. New initiatives are required to seek novel target sites and to further understand insecticide toxicity. One approach to this is to look at mechanisms to regulate xenobiotic uptake in cells.

P-glycoproteins (P-gps) are membrane-spanning proteins from the superfamily of ATP-Binding Cassettes (ABC transporters), which pump molecules out of cells by an ATP-dependent mechanism (Germann & Chambers, 1998). These proteins have been associated with resistance to a number of drugs, particularly resistance to cancer therapy drugs (Juliano & Ling, 1976). Multi-drug resistance (MDR) in tumours is conferred by the overproduction of P-gp by up to 50-fold (e.g. Wang *et al.*, 1995). The net effect of this overproduction is an increased efflux of the drug. Although the normal role of the protein is not clear, what is certain is that P-gp interacts with a large range of chemicals (Germann & Chambers, 1998). This system could well be a first line of defence to the penetration of xenobiotics into the cell.

A protein similar to P-gp has been found in several invertebrate species including *Heliothis, Chironomus* and *Haemonchus* (Lanning *et al.*, 1996a; Podsiadlowski *et al.*, 1998, Xu *et al.*, 1998) and has been shown to interact with environmental xenobiotics, including various pesticides (Bain & LeBlanc, 1996, Lanning *et al.*, 1996 a & b). P-glycoproteins have also been implicated in heavy metal resistance in invertebrates (Callaghan & Beverley, 1991, Broeks *et al.*, 1996). Certain chemicals modulate the interaction between P-gp and xenobiotics. These

"chemosensitisers" may be non-toxic themselves, but through interactions with P-gp can increase the efficacy of more toxic chemicals (Ford, 1996, Smital & Kurelec, 1998). The aim of this study was to determine whether P-gps played a role in insecticide toxicity in *Culex pipiens* mosquitoes.

## MATERIALS AND METHODS

Firstly the presence of P-gp was verified using a specific monoclonal antibody (C219, Georges *et al.*, 1990). Secondly, the interaction between P-gp and pesticides was investigated using a specific chemosensitiser, verapamil (Tsuruo *et al.*, 1981), and two pesticides, endosulfan and chlorpyrifos.

### Immunoblotting

Protein was extracted using the method of Galgani *et al.*, (1996). Twelve adult *Culex pipiens* were homogenised at 4 °C in 200µl lysis buffer (10mM KCl, 1.5 mM $MgCl_2$, 10mM Tris HCl pH 7.4, 2 mM phenyl methyl sulphonyl fluoride, 5% sodium dodecyl sulphate) and sonicated for 15 seconds, 6 micron wave peak (MSE, UK). Debris was removed by centrifugation at 10,000 g for 10 min, 4°C and the supernatant was stored at -70°C. The extract was assayed for total protein using a commercial kit (BCA Assay Kit, Pierce).

Three hundred micrograms of protein extract was boiled for 5 min with an equal volume of sample buffer (50 mM Tris pH 6.8, 10% sucrose, 2.5% β-mercaptoethanol, 0.001% bromophenol blue). Gel electrophoresis was performed on a 6% SDS-polyacrylamide gel as described by Laemmli (1968) using an Atto 6450 minigel system (Atto Corporation, Japan) at 130V, 4°C for 4 h. Proteins were transferred to nitrocellulose membranes (Hybond-ECL, Amersham Pharmacia, UK) using a semi-dry blotter (Biotech Instruments Ltd., Luton, UK), 100mA for 4 h.

Immunodetection was based on the ECL protocol recommended by Amersham Pharmacia using PBS-T buffer. After immersion in a blocking solution (5% Marvel milk powder in PBS-T) overnight at 4°C, membranes were rinsed and washed twice in PBS-T and then incubated with the p-glycoprotein monoclonal antibody C219 (ID Labs Inc., Glasgow, UK). C219 was diluted 1:1000 in PBS-T and 5 ml incubated with the membrane for 2 h. After further washes in PBS/T membranes were incubated with goat anti-mouse antibody conjugated to horseradish peroxidase (Sigma, Poole, UK) at a dilution of 1:3500 in PBS-T with 5% Marvel. The bound antibody was then visualised by electrochemiluminescence (ECL) using reagents and protocols from Amersham Pharmacia.

To verify the system, protein was extracted from *Chironomus riparius* and a cell culture known to overexpress P-gp (Podsiadlowski *et al.*, 1998, Sharp *et al.*, 1994).

### Larval bioassays

Assays were conducted on 4th instar larvae of a laboratory reared strain of *C. pipiens* using standard methods (WHO, 1981). Stock solutions of verapamil HCl (Sigma, Poole, UK) and

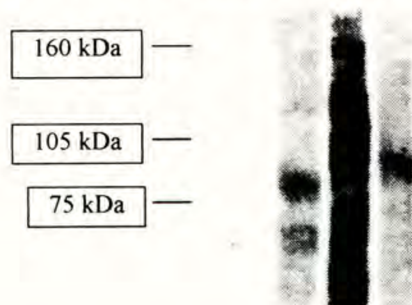
the pesticides (chlorpyrifos, 100% grade, Dow Chemical Company Ltd., King's Lynn, endosulfan, 99.8%, ChemService, Chester, USA) were dissolved in acetone.

Two replicates of 20 larvae in 200 ml distilled water were subjected to a range of doses of the test pesticide with or without 100 μM verapamil HCl, all larvae receiving the same total dose of acetone (1.25 ml). Controls of acetone alone and with verapamil were included. Mortality was assessed after 24 h.

## RESULTS AND DISCUSSION

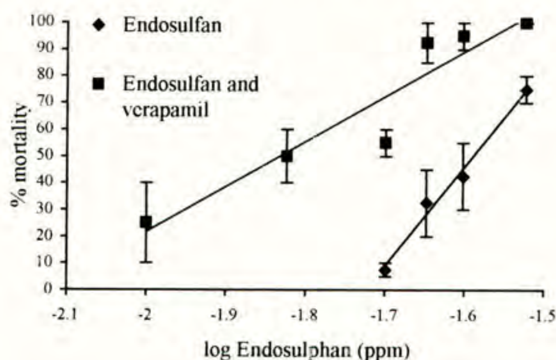
The P-gp antibody C219 detected a protein at ~ 85 kDa, with a fainter band at approximately 65 kDa, demonstrating for the first time the presence of P-gps in mosquitoes (Figure 1). This is smaller than the size normally found in P-gps, (approximately 140 kDa to 170 kDa) though proteins in the range of 110-230 kDa have been found (Smital & Kurelec 1998). Interestingly, the dipteran *C. riparius* was also shown to have a similar size band to the mosquito. *C. riparius* is known to possess P-gp (Podsiadlowski *et al.*, 1998), but this is the first western blot to be published. The presence of two bands raises the possibility of damage during preparation through sonication, though the method was based on a published system for the extraction of P-gp from mussel gills (Galgani *et al.*, 1996). P-gps in humans consist of two homologous halves, of 64 and 88 kDa, which are both capable of ATPase activity (Loo & Clarke, 1994). It is therefore likely that the 75-80 kDa bands observed in Dipteran western blots with the C219 antibody represent half of the P-gp protein.

Figure 1. Immunoblot of PAGE gel probed with monoclonal antibody C219. Lane 1 is *Culex pipiens*. Lane 2 is from cells over expressing P-gp. Lane 3 is *Chironomus riparius*



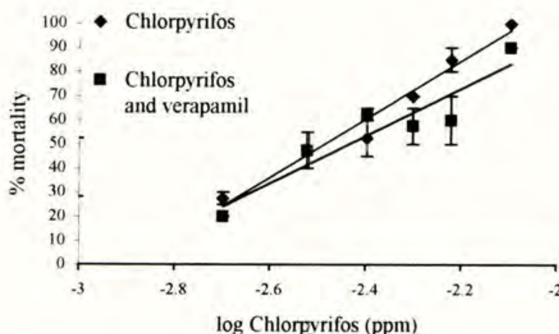

The P-gp modulator verapamil increased endosulfan toxicity approximately 1.6-fold ($LD_{50}$ 0.026 ppm vs. 0.016 ppm) (Figure 2), but had no effect on chlorpyrifos (Figure 3). Verapamil alone was non-toxic. This agrees with work done on cell cultures, which suggests that endosulfan is a P-gp substrate (Bain & LeBlanc 1996, K.G. I. Jayawardena, personal communication) whilst chlorpyrifos is not (Lanning *et al.*, 1996b).

Figure 2. Effect of P-gp inhibitor verapamil on endosulfan larval toxicity of *Culex pipiens*




In summary, mosquitoes have been found to contain p-glycoproteins capable of removing pesticides from insect cells. A P-gp modulator, verapamil, was not toxic to mosquitoes but increased toxicity of endosulfan. It has also been shown to increase the toxicity of cypermethrin and ivermectin (unpublished results). This raises the possibility of using P-gp chemosensitisers to increase pesticide toxicity, or to permit the use of lower concentrations for the same effect.

Figure 3. Effect of P-gp inhibitor verapamil on chlorpyrifos larval toxicity of *Culex pipiens*




Ideally, a P-gp chemosensitiser would be species-specific so that non-target organisms were subjected to lower doses of the insecticide. However, since P-gps are found widely throughout the animal and bacterial kingdoms, this may be impossible to achieve and target specificity will have to be found by other means. Female mosquitoes are, of course, blood feeders and *C. pipiens* mosquitoes are a serious biting nuisance in many parts of the world. They are also important vectors of filariasis and many viruses, including the West Nile virus currently spreading in New York. The anthelminthic drug ivermectin is used in tropical areas to control onchoceriasis and filariasis and the WHO is preparing a world-wide program to eliminate lymphatic filariasis by annual treatment of a billion infected people (WHO 1997). It is possible

that malarial drugs, also shown to be P-gp substrates, are modulating P-gp (Peel *et al.*, 1994). Intriguingly both ivermectin and the standard antimalarial drug quinine have been investigated clinically as chemosensitisers for cancer treatment (Pouliot *et al.*, 1997; Solary *et al.*, 1991). Since ivermectin interacts with mosquito P-gp, increasing the toxicity of insecticides that are P-gp substrates, there is a possibility of co-ordinating clinical and pesticide programs to improve vector control, i.e. by use of insecticides that are known P-gp substrates. However, if these drugs have a severe effect on mosquito fitness, it is possible that mosquito strains will be selected for increased P-gp expression or particular alleles, which could lead to resistance.

## ACKNOWLEDGEMENTS

We thank Dr. Jayawardena for his assistance.

## REFERENCES

Bain L J; LeBlanc G A (1996). Interaction of structurally diverse pesticides with the human *MDR1* gene product p-glycoprotein. *Toxicology and Applied Pharmacology* **141**, 288-298.

Broeks A; Gerrard B; Allikmets R; Dean M; Plasterk RH (1996). Homologues of the human multidrug resistance genes MRP and MDR contribute to heavy metal resistance in the soil nematode *Caenorhabditis elegans*. *EMBO Journal* **22** 6132-43

Callaghan H L; Beverley S M (1991) Heavy metal resistance: a new role for P-glycoproteins in *Leishmania*. *Journal of Biological Chemistry* **266** 18427-30

Ford J M (1996). Modulators of multidrug resistance. Preclinical studies. *Hematological Oncological Clinical North America* **9** 337-361.

Galgani F; Cornwall R; Toomey B H; Epel D D (1996). Interaction of environmental xenobiotics with a multixenobiotic defence mechanism in the bay mussel *Mytilus galloprovincialis* from the coast of California.. *Environmental Toxicology and Chemistry* **15**, 325-331.

Georges E; Bradley G; Gariepy J; Ling V (1990). Detection of P-glycoprotein isoforms by gene-specific monoclonal antibodies. *Proceedings of the National Academy Science U S A* **87:1** 152-6.

Germann U A; Chambers T C (1998). Molecular analysis of the multidrug transporter, p-glycoprotein. *Cytotechnology* **27**, 31-60.

Juliano R L; Ling V (1976). A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochimica Biophysica Acta* **455**, 152-162.

Laemmli U K (1968). Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature* **227**, 680-685.

Lanning C L; Fine R L; Corcoran J J; Ayad H M; Rose R L; Abou-Donia M B (1996a). Tobacco budworm p-glycoprotein; biochemical characterisation and its involvement in pesticide resistance. *Biochemica Biophysica Acta* **1291**, 155-162.

Lanning C L; Fine R L; Sachs C W; Rao U S; Corcoran J J; Abou-Donia M B (1996b). Chlorpyrifos oxon interacts with the mammalian multidrug resistance protein p-glycoprotein. *Journal of Toxicology and Environmental Health* **47**, 395-407.

Loo T W; Clarke D M (1994). Reconstitution of drug-stimulated ATPase activity following co-expression of each half of human P-glycoprotein as separate polypeptides. *Journal of Biological Chemistry* **269,** 7750-5.

Peel S A; Bright P; Yount B; Handy J; Baric R S (1994). A strong association between mefloquine and halofantrine resistance and amplification, overexpression, and mutation in the P-glycoprotein gene homolog (pfmdr) of *Plasmodium falciparum in vitro. American Journal of Tropical Medicine* **51**, 648-58.

Podsiadlowski L; Matha V; Vilcinskas A (1998). Detection of a p-glycoprotein related pump in *Chironomus* larvae and its inhibition by verapamil and cyclosporin A. *Comparative Biochemistry and Physiology B* **121**, 443-450.

Pouliot J F; L'Heureux F L; Liu Z; Prichard R K; Georges E (1997). Reversal of p-glycoprotein associated multidrug resistance by ivermectin. *Biochemical Pharmacology* **53,** 17-25.

Sharp S Y; Rowlands M G; Jarman M; Kelland L R (1994). Effects of a new antioestrogen, idoxofene. on cisplatin- and doxorubicin-sensitive and -resistant human ovarian carcinoma cell lines. *British Journal of Cancer* **70**, 409-414.

Smital T; Kurelec B (1998). The chemosensitisers of multixenobiotic resistance mechanism in aquatic invertebrates: a new class of pollutants. *Mutation Research* **399**, 43-53.

Solary E; Velay I; Chauffert B; Bidan J M; Caillot D; Dumas M; Guy H (1991). Sufficient levels of quinine in the serum circumvent the multidrug resistance of the human leukemic cell line K562. *Cancer* **68,** 1717-1719.

Tsuruo T; Iida H; Tsukagoshi S; Sakurai Y (1981). Overcoming of vincristine resistance in P388 leukaemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Research* **41,** 1967-72.

Wang S J; Scavetta R; Lenz H J; Danenburg K; Danenburg P V; Schonthal A H (1995). Gene amplification and multidrug resistance induced by the phosphatase-inhibitory tumour promoter okadeic acid. *Carcinogenesis* **16,** 637-641.

WHO (1981). Instructions for determining the susceptibility or resistance of mosquito larvae to insecticides. WHO/VBC, 81.807. World Health Organisation.

WHO (1997). Lymphatic filarisasis: reasons for hope. Document WHO/CTD/Fil97.4 Rev.1.

Xu M; Molento M; Blackhall W J; Ribiero P; Beech R N; Pritchard R K (1998). Ivermectin resistance in nematodes may be caused by alteration of p-glycoprotein homologue. *Molecular and Biochemical Parasitology* **91**, 327-335.

# A fluorometric method to detect insensitive acetylcholinesterase in resistant pests

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## ABSTRACT

A sensitive method for monitoring the presence of modified acetylcholinesterase insensitive to organophosphorus (OP) or carbamate insecticides in individual insects and mites is described. This method readily characterises AChE sensitivity from individual tobacco whitefly (*Bemisia tabaci*) and, for the first time, individual two-spotted spider mite (*Tetranychus urticae*). Groups of individuals from various locations were examined for the presence of this target-site resistance mechanism. Findings include a novel, insensitive AChE variant in a population of *Bemisia tabaci* from Arizona, and an apparent uniformity of the insensitive enzyme in mite populations world wide.

## INTRODUCTION

Acetylcholinesterase (AChE) is the target-site for organophosphorus and carbamate insecticides. Modifications to the enzyme that result in insensitivity have been found in many insect species (Hama, 1983) and even within an insect species several different variants conferring varying insensitivity profiles have been identified (Devonshire & Moores, 1984, Byrne & Devonshire, 1993). Biochemical tests provide a powerful means to monitor for resistance, with a capability to distinguish between resistant individual heterozygotes and homozygotes for resistance genes. Although a microtitre-plate assay has been described for characterising insensitive AChE in individual insects using conventional spectrophotometry (Moores *et al.*, 1988), limitations on its sensitivity has meant the number of inhibitors used to build an insensitivity profile of the sample may be very limited for small insects. Assays on individual mites or insects such as whitefly, where even the uninhibited AChE activity is relatively low, were either not possible or lengthy, drastically reducing the high-throughput of the technique, and so compromising one of the great advantages of using the biochemical assay to monitor for resistance genotypes.

We report here on a more sensitive fluorometric assay that is capable, for the first time, of identifying AChE sensitivity from individual two-spotted spider mite (*Tetranychus urticae*). We also identify a novel, insensitive AChE variant from a strain of tobacco whitefly (*Bemisia tabaci*).

## MATERIALS AND METHODS

### Strains of *Bemisia tabaci*

The strains of *B. tabaci* used in this study included a laboratory reference strain that is susceptible to insecticides (SUD-S) and a further three strains from diverse geographical locations and hosts, as summarised in Table 1. All cultures were reared at IACR-Rothamsted without insecticide selection on cotton plants (*Gossypium hirsutum*) in a 16:8 h light:dark regime at temperatures of 28°C (day) and 24°C (night).

Table 1. Strains of *Bemisia tabaci* used to study AChE genotypes.

| Strain | Origin | Host | Received | AChE genotype | Reference |
|---|---|---|---|---|---|
| SUD-S | Sudan | Cotton | 1978 | Sensitive | Byrne & Devonshire, 1993 |
| ISR-R | Israel | Cotton | 1991 | Type 1 insensitive | Byrne *et al.* 1994 |
| PAK-9 | Pakistan | Aubergine | 1994 | Unknown | - |
| MCK | Arizona | Poinsettia | 2000 | Unknown | - |

### Strains of *Tetranychus urticae*

The laboratory susceptible strain (GSS) was originally provided by Schering AG, Berlin, having been maintained in laboratory culture without acaricide treatment since 1965. The OP-resistant strain WIESMOOR (WI) was treated with the OP oxydemeton-methyl (0.3g/litre) twice a year and has been maintained in the laboratory at Bayer AG since 1954. Origin, host and the year of collection for all other strains are given in Table 2. All strains of *T. urticae* were maintained on French beans (*Phaseolus vulgaris*) at 24-25°C, a photoperiod of 16:8 h (light:dark) and 50-60% relative humidity. For all strains the sensitivity of the AChE was unknown.

Table 2. Strains of *Tetranychus urticae* used to study AChE genotypes

| Strain | Origin | Host | Received |
|---|---|---|---|
| GSS | Germany | Bean | 1998 |
| WIESMOOR (WI) | Germany | Cucumber | 1954 |
| AKITA | Japan | Ornamentals | 1996 |
| I-98 | Italy | Bean | 1998 |
| CA-98 | California | Almond | 1998 |
| GR-99 | Greece | Bean | 1999 |
| NL-00 | Netherlands | Rose | 2000 |
| SA1-00 | South Africa | Rose | 2000 |
| BR4-00 | Brazil | Rose | 2000 |

### Assay Procedure

Inhibition of AChE in individuals was measured as described by Moores *et al.* (1988) but using a fluorometric assay with slight modifications according to Parvari *et al.* (1983). Briefly, the hydrolysis of the substrate acetylthiocholine iodide (ATChI) was measured fluorometrically by the emission of a fluorescent product formed by the reaction of N-(4-(7-diethylamino-4-methylcoumarin-3-yl)phenyl)maleimide (CPM) with the liberated thiocholine, to give a reaction product with an intense blue fluorescence. Up to 96 individuals were homogenised in separate wells of a microtitre plate (NUNC) in 5 µl of 0.1 M phosphate buffer, pH 7.5 containing 0.1% Triton X-100 using a multihomogeniser (Moores *et al.*, 1988). After dilution to 80 µl with the same buffer and leaving for 10 min to solubilise, replicate aliquots, equivalent to 0.25 of a whitefly or spider mite, were assayed continuously in fluorometric microtitre plates (Perkin-Elmer, Solid White) for 20 min in the absence and presence of diagnostic concentrations of inhibitor. The excitation wavelength was 390 nm (slit width 5 nm) and the emission wavelength set to 473 nm. Final substrate and CPM concentrations were 0.5 mM and 1.0 µM respectively. In order to limit fluorescence caused by non-enzymatic substrate hydrolysis, ATChI solutions were always freshly prepared. Data were analysed and presented as by Moores *et al.* (1988).

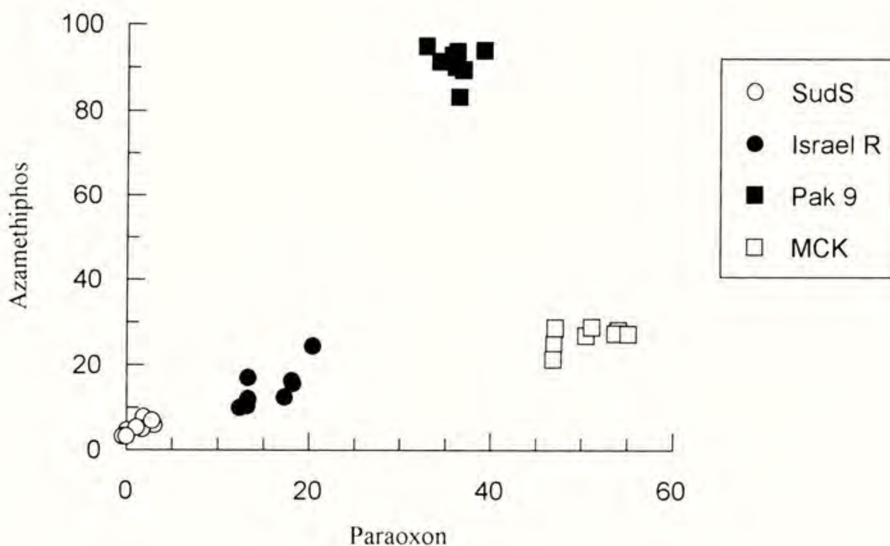


Fig. 1. Bivariate plot of mean % activity remaining during inhibition of AChE from strains of *B. tabaci* by 10 µM azamethiphos and 100 µM paraoxon.

Individual whiteflies were characterised by adding three equal fractions (20µl) of homogenate to substrate and CPM with and without discriminating inhibitor concentrations of paraoxon (100 µM) or azamethiphos (10 µM). Enzyme activity was measured on a LS50B luminescence spectrometer (Perkin-Elmer).

Individual mites were characterised by adding three equal fractions (20µl) of the homogenate to buffer or discriminating inhibitor concentrations of chlorpyrifos-oxon (0.1 µM) and ethyl paraoxon (10 µM). Enzyme and inhibitor were preincubated for 20 min before adding substrate and CPM as this was found to optimise the resolution of the various AChE forms. Enzyme activity was assayed with a Polarion spectrofluorometer (Tecan).

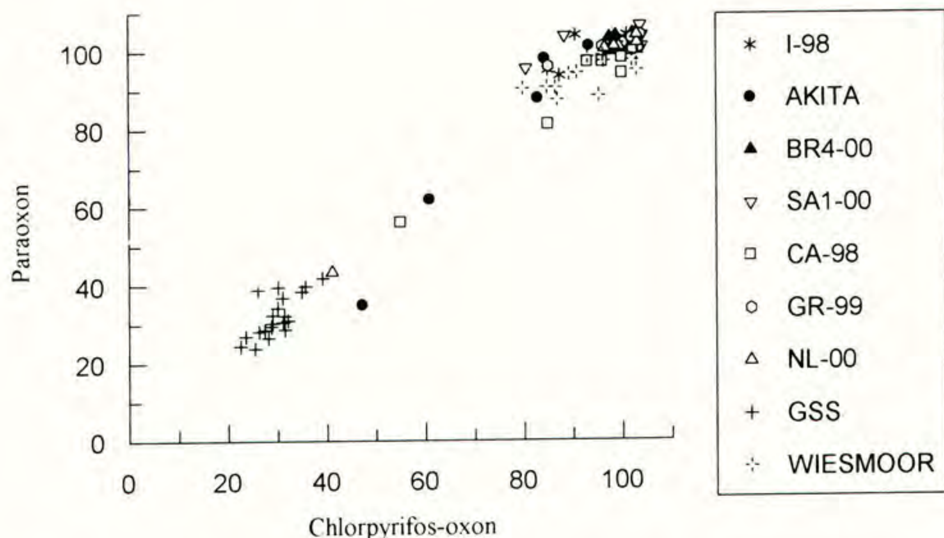


Fig. 2. Bivariate plot of mean % activity remaining during inhibition of AChE from strains of *T. urticae* by 0.1µM chlorpyrifos-oxon and 10 µM paraoxon.

## RESULTS AND DISCUSSION

### *Bemisia tabaci*

Two insensitive forms of AChE have been recorded previously in *B. tabaci* both of which showed paraoxon insensitivity, and one of which also demonstrated considerable insensitivity to azamethiphos (Byrne & Devonshire, 1993). The AChE variant (ISR-R) that demonstrated insensitivity to paraoxon alone was found to exhibit ca. 10-fold resistance in bioassays to chlorpyrifos and 20-fold to monocrotophos, as compared to a second strain with the same esterase background but lacking the insensitive AChE (Byrne *et al.*, 1994). In the present assay, the discriminating dose of

paraoxon was greatly increased as compared to the colorimetric assay, and this allowed a third, novel, insensitive AChE variant to be identified in strain MCK (Fig. 1). The PAK-9 genotype appears to be the same as that recorded earlier in another strain from Pakistan, PAK-1 (Byrne & Devonshire, 1997). The new AChE variant has far greater insensitivity to paraoxon than any previously recorded from *B. tabaci*. Although bioassay data are not yet available for this strain, it may be expected that this very high insensitivity will be reflected in high resistance to OPs. The extent of the measured resistance will also depend on the presence or absence of other metabolic mechanisms.

### *Tetranychus urticae*

Insensitive AChE was first reported in *T. urticae* as long ago as 1964 (Smissaert, 1964). However, this is the first time that an assay has been recorded that is sensitive enough to give information about the AChE content of an individual mite. The ability to characterise single mites is crucial for identifying AChE genotypes that may be present in low frequencies within a population.
Bivariate plots of AChE activity in the presence of two inhibitors expressed as a percentage of the uninhibited activity clearly revealed the insensitivity profiles of the strains (Fig. 2). Only one form of insensitive AChE has been identified in this species to date, giving tolerance to both paraoxon and chlorpyrifos-oxon. On examination of individuals from the standard strain of spider mite, GSS was homogeneous for the fully sensitive AChE genotype and the Wiesmoor strain, kept under constant selection, was fully homogeneous for the insensitive AChE genotype. Our finding that all individuals examined from diverse global regions fell on a line between these genotypes suggests that they contained the same genes, either homozygous or heterozygous. All strains were homogeneous for the insensitive AChE except; NL-00 had a single individual homozygous for the sensitive genotype, CA-98 had a single heterozygous individual, and AKITA had a single heterozygous and a single homozygous sensitive individual. This predominance of the insensitive AChE probably reflects the heavy use of OPs that have been used against spider mites for many decades. The fact that it appears in such diverse geographical locations suggests that the genotype arose once a long time ago and then spread through populations, or it is a relatively frequent mutational event. This finding of a single insensitive form of AChE is also supported by a more detailed investigation of AChE genotypes carried out by Stumpf *et al.* (2000) which includes kinetic characterisation of sensitive and insensitive forms and corresponding bioassay data.

## ACKNOWLEDGEMENTS

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## REFERENCES


Byrne F J; Devonshire A L (1993). Insensitive acetylcholinesterase and esterase polymorphism in susceptible and resistant populations of the tobacco whitefly *Bemisia tabaci* (Genn). *Pesticide Biochemistry and Physiology*. **45**, 34-42.

Byrne F J; Devonshire A L (1997). Kinetics of insensitive acetylcholinesterases in organophosphate-resistant tobacco whitefly, *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae). *Pesticide Biochemistry and Physiology*. **58**, 119-124.

Byrne F J; Cahill M; Denholm I; Devonshire A L (1994). A biological and toxicological study of the role of insensitive acetylcholinesterase in organophosphorus resistant *Bemisia tabaci* (Homoptera: Aleyrodidae) from Israel. *Bulletin of Entomological Research*. **84**, 179-184.

Devonshire A L; Moores G D (1984). Different forms of insensitive acetylcholinesterase in insecticide-resistant house flies (*Musca domestica*). *Pesticide Biochemistry and Physiology*. **21,** 336-344.

Hama H (1983). Resistance to insecticides due to reduced sensitivity of acetylcholinesterase. In: *Pest resistance to insecticides*, eds. G P Georghiou & T Saito, pp. 299-331. Plenum Press: New York.

Moores G D; Devonshire A L; Denholm I (1988). A microtitre plate assay for characterising insensitive acetylcholinesterase genotypes of insecticide-resistant insects. *Bulletin of Entomological Research*. **78**, 537-544.

Parvari R; Pecht I; Soreq H (1983). A micro-fluorometric assay for cholinesterases, suitable for multiple kinetic determinations of picomoles of released thiocholine. *Analytical Biochemistry*. **133**, 450-456.

Smissaert H R (1964). Cholinesterase inhibition in spider mites susceptible and resistant to organophosphates. *Science*. **143**, 129-131.

Stumpf N; Nauen R; Moores G D; Zebitz C P W; Kraus W (2000). Resistance to organophosphates and biochemical genotyping of acetylcholinesterase in *Tetranychus urticae* (Acari: Tetranychidae). *Pesticide Biochemistry and Physiology*. (Submitted).

# Efficacy of BAJ 2740, a new acaricidal tetronic acid derivative, against tetranychid spider mite species resistant to conventional acaricides

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## ABSTRACT

BAJ 2740 (ISO proposed common name: spirodiclofen) is a new acaricidal compound particularly active against spider mites, e.g. the two-spotted spider mite, *Tetranychus urticae*, and the European red mite, *Panonychus ulmi*. BAJ 2740 is a phenyl-substituted spirocyclic tetronic acid derivative, i.e. it belongs to a new class of chemistry. The compound is active against eggs, larvae, nymphs, all quiescent stages, and adult females. The baseline susceptibilities of several strains of *T. urticae* and *P. ulmi* were similar as shown by a larvae spray bioassay. BAJ 2740 was fully active to several strains of *T. urticae* showing resistance to organophosphates, hexythiazox, dicofol, clofentezine, pyridaben, fenpyroximate, abamectin and others. Additionally cross-resistance to organophosphates, hexythiazox and clofentezine was not detected in *P. ulmi*. Furthermore a field-derived population of *T. urticae* was artificially selected in the laboratory, but even after treatment of 29 generations resistance factors had risen only very moderately in this population.

## INTRODUCTION

Phytophagous mites such as the two-spotted spider mite, *Tetranychus urticae*, and the European red mite, *Panonychus ulmi*, are responsible for significant yield losses in many important agronomic crops worldwide (Jeppson *et al.*, 1975). The application of acaricides is usually required to maintain mite populations below economic thresholds, but widespread resistance to several traditional classes of acaricides enables acaricidal compounds with new modes of action rapidly to become part of resistance management programs. The study undertaken here describes the establishment of a suitable bioassay procedure, the evaluation of baseline susceptibility data for more than a dozen strains of *T. urticae* and *P. ulmi* from several countries, and the resistance risk assessed by artificial selection pressure for BAJ 2740, a novel broad spectrum acaricide (Wachendorff *et al.*, 2000).

## MATERIALS AND METHODS

### Spider mite strains

All strains of *T. urticae* and *P. ulmi* (Acari: Tetranychidae) used throughout the study are listed in Table 1. *T. urticae* and *P. ulmi* were maintained under greenhouse conditions at 24-25°C, a photoperiod of 16:8 hours (L:D) and 50-60 % relative humidity on French beans, *Phaseolus vulgaris*, and on plum, *Prunus domestica*, respectively. The mitochondrial electron transport inhibitor (METI) resistance in strain AK as well as the resistance pattern of some of the other

strains used was described recently by Nauen *et al.* (2000). Field strain NL1-00 showed a 56-fold resistance to abamectin (unpublished). Strain UKMR was kindly provided by IACR Rothamsted and showed a strong resistance to METI acaricides (G. Devine, *pers. comm.*).

### Acaricides

BAJ 2740 (ISO proposed common name: spirodiclofen) was of technical grade of the highest purity available. Stock solutions were prepared by dissolving 100 mg active ingredient in 1 ml of a 3:1 (v/v) mixture of N,N-dimethylformamide and alkylarylpolyglykolether (emulgator) and then diluted 100-fold with water resulting in an acaricide start concentration of 1 g/litre.

Table 1. Strains of *Tetranychus urticae* and *Panonychus ulmi* tested

| Strain | Origin | Host | Received | Known resistance |
|---|---|---|---|---|
| ***Tetranychus urticae*** | | | | |
| GSS | Germany | Bean | 1998 | Susceptible |
| WI | Germany | Cucumber | 1954 | OP's (laboratory selected) |
| AU | Australia | Rose | 1987 | Hexythiazox, clofentezine |
| AK | Japan | Ornamentals | 1996 | METIs |
| VB-98 | USA | Cotton | 1998 | OP's |
| IT-98 | Italy | Bean | 1998 | Low-level, several a.i.'s |
| UKMR | UK | Hop | 1999 | METIs |
| GR-99 | Greece | Cotton | 1999 | Not determined |
| NL1-00 | Netherlands | Rose | 2000 | Abamectin |
| BR1-00 | Brazil | Cotton | 2000 | Not determined |
| BR2-00 | Brazil | Cotton | 2000 | Not determined |
| BR3-00 | Brazil | Chrysanthemum | 2000 | Not determined |
| SA1-00 | South Africa | Rose | 2000 | Not determined |
| ***Panonychus ulmi*** | | | | |
| HS | Germany | Apple | 1990 | Susceptible |
| CE | France | Apple | 1991 | Hexythiazox, clofentezine |
| US | USA | Apple | 1997 | OP's |

### Bioassays

In order to assess the susceptibility of each developmental stage of *T. urticae* the following test procedure was established; Sticky ring-shaped barriers (4 cm in diameter) were applied to a single remaining leaf of defoliated 14-21 d old French bean plants. By means of a pencil thirty adult females were placed within the sticky barrier and allowed to lay eggs. After 4 h females were removed. Treatments with aqueous solutions of different concentrations of BAJ 2740 a.i. were performed by spray application (100 ml) to the desired developmental stage (Table 2). Each bioassay consists of at least five concentrations (2 replicates) and was repeated twice. After hatching of the larvae, each subsequent developmental stage lasts c. 1 d until adulthood is reached (7-8 days at 23 ± 1°C).

Table 2. Developmental stages of *Tetranychus urticae*

| No. | Stage | Mobile |
|---|---|---|
| 1 | Egg | No |
| 2 | Larva | Yes |
| 3 | Nymphochrysalis | No (quiescent) |
| 4 | Protonymph | Yes |
| 5 | Deutochrysalis | No (quiescent) |
| 6 | Deutonymph | Yes |
| 7 | Teleiochrysalis | No (quiescent) |
| 8 | Adult | Yes |

Two days after treatment the status of development and the number of individuals within the sticky barrier were counted for the first time. The second assessment took place after the appearance of the first adults on control plants treated with solvent only. Log-dose mortality data were subjected to probit regression analysis to calculate $LC_{50}$-values (POLO PC, LeOra Software, Berkeley, USA).

All resistance bioassays were performed on larvae directly after egg-hatch as described elsewhere (Nauen *et al.*, 2000). Briefly, eight adult females of *T. urticae* were transferred to 14 day old French bean plant as described above. Oviposition was terminated by removing all females after 20 h at 22 ± 1°C and RH 60-70%. After 7 d 25-40 larvae hatched from the eggs laid and plants were then immediately treated by spray-application as described above. One day after treatment the total number of larvae and nymphochrysalis was scored, and the final assessment of mortality was performed after the appearance of adults on the solvent-treated control plants (usually 7-8 d after treatment). Control mortality never exceeded 10%.

The resistance bioassay procedure for *P. ulmi* was comparable to the methodology described for *T. urticae*. Seven week-old plum plants (*Prunus domestica*) were cut down to 25-30 cm and all leaves in excess of three were removed. With the aid of a plastic ring a circular sticky barrier (4 cm in diameter) was applied onto the upper surface of each leaf. Fifteen adult females were allowed to lay 25-40 eggs within each circular area for 24 hours before removal. All subsequent steps as well as the bioassay conditions were the same as described above for *T. urticae*.

Artificial selection for resistance to BAJ 2740 was undertaken using strain IT-98, which had shown low level resistance to several acaricides when treated with diagnostic concentrations (Nauen *et al.*, 2000). IT-98 was treated as a mixed population on French bean plants by spray application with BAJ 2740 every generation using concentrations between 0.5 and 10 mg/litre.

## RESULTS AND DISCUSSION

### Efficacy of BAJ 2740 to developmental stages of *T. urticae*

In order to assess the efficacy of BAJ 2740 against the different developmental stages of *T. urticae* a simple and reliable bioassay was chosen where mites on French bean leaves were sprayed with aqueous laboratory formulations. BAJ 2740 was active against all developmental stages (Figure 1). The $LC_{50}$ values determined for the different stages ranged roughly between

0.1 and 1 mg/litre. Slope values were high in general providing $LC_{90}$ values often close to the $LC_{50}$ values (not shown). The ovicidal effects were excellent and seem to be independent of age, i.e. 2 d and 4 d old eggs showed the same susceptibility towards BAJ 2740. Teleiochrysalis, the last quiescent stage before adult moulting, was next to adults the least susceptible stage.

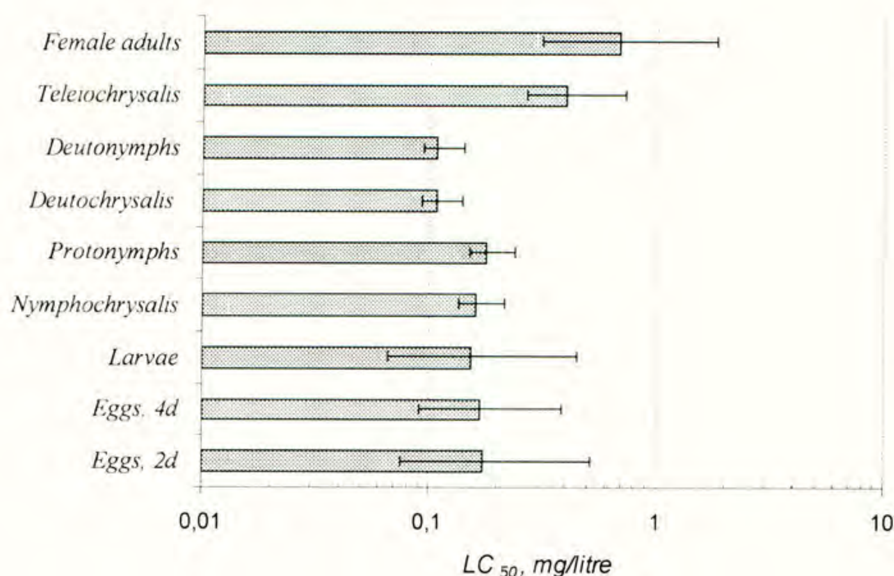


Figure 1. Efficacy ($LC_{50}$ value) of BAJ 2740 against developmental stages of *Tetranychus urticae* (strain WI) 7d after spray application on French bean leaves

According to these results larvae were chosen as the principal stage for the determination of baseline susceptibility of several strains of *T. urticae* collected from all over the world. *P.ulmi*, another important tetranychid spider mite pest, behaved in a similar way (data not shown), and in this case also the six-legged larval stage was chosen for resistance bioassays.

**Baseline susceptibility of *T. urticae* and *P. ulmi***

The variation in response to BAJ 2740 between larvae of all *T. urticae* strains, investigated as measured by the $LC_{50}$ value of log-dose percentage-mortality lines (Figure 2), was between 0.1 (strain WI) and 1.1 mg/litre (strain BR3-00). An overall composite (all data combined) $LC_{50}$ value of 0.33 and 0.36 mg/litre for *T. urticae* and *P. ulmi*, respectively, was estimated in order to serve as a basis to assess the susceptibility of field populations going to be monitored after the official launch of the new acaricide BAJ 2740 (Table 3). The corresponding $LC_{99}$ values for BAJ 2740 against larvae of *T. urticae* and *P. ulmi* were 3.9 and 1.8 mg/litre, respectively, and will be used as discriminating doses to monitor field populations of both species for BAJ 2740 resistance in the future. Taking all strains of *T. urticae* together the mean percentage mortality at 1.6 and 8 mg/litre was 94.2 and 100 %, respectively. The recommended field dose for *T.*

*urticae* (Wachendorff *et al.*, 2000) is at least 25 times above the $LC_{99}$ against larvae determined in this study, and may therefore also provide excellent control of more robust phenotypes such as strain BR3-00.

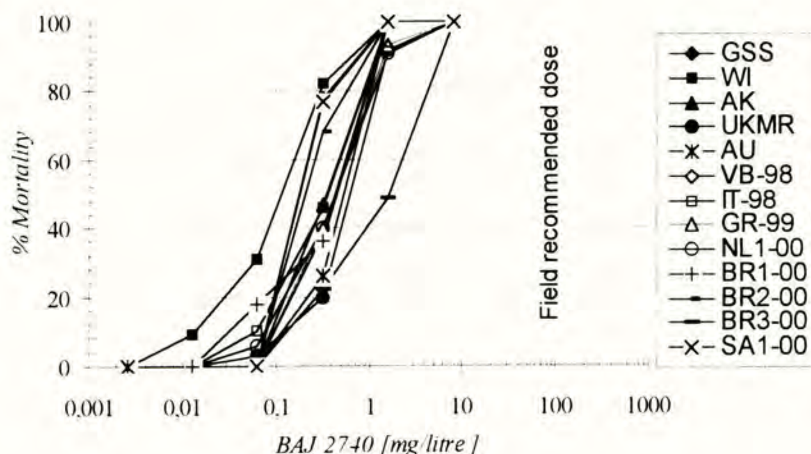


Figure 2. Dose-response relationship for BAJ 2740 against larvae of different laboratory and field-collected strains of *Tetranychus urticae*

No cross-resistance to pyridaben, fenpyroximate, tebufenpyrad, abamectin, hexythiazox, clofentezine, dicofol and organophosphates was detected in several strains of *T. urticae* and *P. ulmi* tested in this study (Table 1).

Table 3. Estimation of a composite $LC_{50}$ value using the baseline data of all strains tested

| Species | Number of strains combined | Composite $LC_{50}$ [mg/litre] | Fiducial limits 95% | Slope ± SE |
|---|---|---|---|---|
| *T. urticae* | 13 | 0.33 | 0.23 - 0.47 | 2.1 ± 0.18 |
| *P. ulmi* | 3 | 0.36 | 0.31 - 0.43 | 3.4 ± 0.38 |

**Resistance risk assessment**

The continuous artificial selection of a former *T. urticae* field population from Italy (strain IT-98) with BAJ 2740 by spray application resulted in a very slow increase of resistance factors. The parental generation of IT-98 showed an $LC_{50}$ value of 0.28 mg/litre when the selection experiment was started. This value coincides very well with the $LC_{50}$ value of 0.33 mg/litre determined as the composite baseline response of *T. urticae* against BAJ 2740. After 29 selection cycles the $F_{29}$ generation showed an $LC_{50}$ value of 1.57 (FL95% 1.29-1.92), thus exhibiting a resistance factor of c. 5 (Figure 3). However, although the resistance factor is very low it can be regarded as significant considering the 95% fiducial limits. The computed $LC_{99}$

value was 23.6 mg/litre in this selected strain, i.e. c. 6 times higher than the composite $LC_{99}$ (3.9 mg/litre) derived after combining the baseline data of all strains investigated. However, this concentration-value is still below the recommended application rates (Wachendorff *et al.*, 2000). On the one hand, the results from the selection experiment resulted in a moderate increase of resistance (5-fold resistance after 29 applications), but still within the range of susceptibility variation observed for field strains of *T. urticae*. On the other hand, the conducted selection experiment suggested that BAJ 2740 - if used carefully and as recommended for several crops (one application per cropping cycle) - will be an excellent chemical partner in resistance management strategies, with low tendency to select rapidly for resistance in tetranychid spider mite pest species.

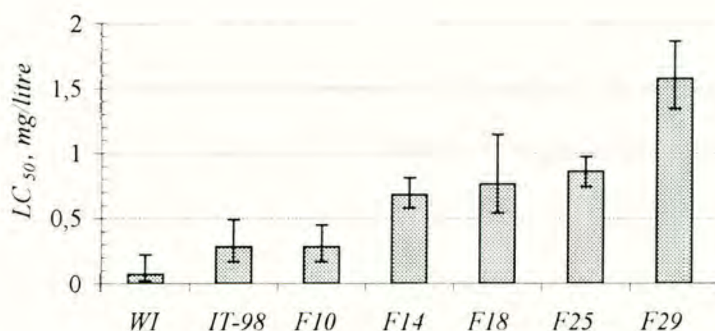


Figure 3. $LC_{50}$ values of continuously laboratory-selected *Tetranychus urticae* (strain IT-98, parental generation) in comparison to a non-selected population of strain WI against BAJ 2740


## ACKNOWLEDGEMENTS

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## REFERENCES


Jeppson L R; Keifer H H; Baker E W (1975). *Mites injurious to economic plants*. University of California Press: Berkeley, CA.

Nauen R; Stumpf N; Elbert A; Zebitz C P W; Kraus W (2000). Acaricide toxicity and resistance in larvae of different strains of *Tetranychus urticae* and *Panonychus ulmi* (Acari: Tetranychidae). *Pest Management Science*, in press.

Wachendorff U; Brueck E; Elbert A; Fischer R; Nauen R; Stumpf N; Tiemann R (2000). BAJ 2740 - A novel broad spectrum acaricide. *Proceedings of the Brighton Crop Protection Conference - Pests and Diseases*

# Status of pesticide resistance in UK populations of the glasshouse whitefly, *Trialeurodes vaporariorum*, and the two-spotted spider mite, *Tetranychus urticae*

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## ABSTRACT

Collections of UK whiteflies (*Trialeurodes vaporariorum*) and spider mites (*Tetranychus urticae*) were examined for resistance to pesticides currently of importance or potential to growers of protected crops. All whitefly strains tested showed resistance (>1000-fold) to the insect growth regulator (IGR) buprofezin. These populations cross-resisted another IGR, teflubenzuron. Over a three-year period, whitefly collections exhibited no tolerance to imidacloprid. Bioassays against spider mites showed one instance of low resistance to abamectin (6-fold) and one case of strong resistance to fenbutatin oxide (40-fold). The first two instances of resistance in Europe to the METI (Mitochondrial Electron Transport Inhibiting) -acaricide tebufenpyrad were also documented, one from a glasshouse chrysanthemum crop and one from a hop garden (c. 40-fold resistance). The use of buprofezin and teflubenzuron in IPM programmes against whitefly is now seriously compromised, and the use of METI-acaricides against spider mites requires careful vigilance to ensure their continued effectiveness.

## INTRODUCTION

Two of the most important arthropod pests in UK glasshouses are the glasshouse whitefly, *Trialeurodes vaporariorum* (Homoptera: Aleyrodidae), and the two-spotted spider mite, *Tetranychus urticae*. (Acari: Tetranychidae). Although both are known to have developed resistance in this country, there has been little recent work on its incidence or its relationship to control practices adopted by growers.

Biological control agents often form effective components of pest management strategies against both these pests, but insecticides retain a crucial role in dealing with unexpected pest outbreaks or for reducing populations to levels at which parasitoids or predators can work more effectively. Only a handful of approved products exist.

In the case of *T. vaporariorum*, the most widely used compound has been the insect growth regulator (IGR) buprofezin. Buprofezin combines a very high intrinsic toxicity against larval stages of susceptible whitefly with little effect upon non-target organisms. Resistance to this compound was first reported in *T. vaporariorum* collections from Holland (De Cock *et al.*, 1995) and in recent years reports of such control failures have become increasingly frequent and widespread. In the case of *T. urticae*, reliable control within IPM programmes in protected edible crops is dependent on the combined use of predatory mites and the selective acaricide, fenbutatin oxide. Other acaricides achieving increasing use on ornamentals are abamectin ('Dynamec') and the METI (mitochondrial electron transport inhibiting) compounds represented by tebufenpyrad ('Masai') and fenazaquin ('Matador'). These three compounds

are perceived by growers as being extremely effective, particularly in light of the fact that many older active ingredients are already strongly resisted. Between 1997 and 2000, we sampled populations of these two species from a large number of glasshouses in England to investigate the current status of resistance and its impact on pest management strategies.

## METHODS AND MATERIALS

### *Trialeurodes vaporariorum*

The standard susceptible (LAB-S) was originally established by L.S. Wardlow in the UK and subsequently reared for ten years in an untreated glasshouse at Cornell University, USA (J. P. Sanderson, pers. comm.). An additional 28 glasshouse samples were collected and tested against buprofezin ('Applaud'), imidacloprid ('Intercept 70WG') and teflubenzuron ('Nemolt'), as well as conventional insecticide classes. All whitefly colonies were maintained on bean plants (*Phaseolus vulgaris*) at 22±3°C. Larval bioassays, on IGRs were performed by testing early instar whitefly nymphs, followed Cahill *et al.*, (1996a). Mortality was assessed when surviving insects had reached late fourth instar; 22-25 days after egg laying. Imidacloprid assays, based on a systemic uptake method (Cahill *et al.*, 1996b) were used to assess adult mortality 72 hours after exposure.

### *Tetranychus urticae*

The standard reference susceptible strain (GSS) originated from Schering AG, Berlin in 1992, and had been in culture since 1965. A Japanese METI-acaricide resistant strain (Akita) was obtained from Bayer AG in 1996. A total of 16 other strains were collected from glasshouses across southern England, mostly from tomato, cucumber and chrysanthemum. All were maintained on bean plants (*Phaseolus vulgaris*) in the laboratory, without further pesticide selection and under constant light conditions at 25±3°C. The main acaricides tested were fenbutatin oxide ('Torque'), abamectin ('Dynamec') and tebufenpyrad ('Masai'). The end point for the assays examining the former was at 72 hrs, so treated mites were held in leaf cells on whole bean plants. METI-acaricides exert their effect very rapidly, so treated mites were held without food in the wells of microtitre plates until scored at 4 hrs. All assays were adaptations of a microimmersion assay developed by Dennehy *et al.*, (1993).

## RESULTS

### *Trialeurodes vaporariorum*

Buprofezin and teflubenzuron

The susceptible strain (LAB-S) had an $LC_{50}$ for buprofezin of approximately 0.01ppm, with 100% kill being achieved at around 1-2 ppm. All UK glasshouse strains showed evidence of resistance to this compound. In some strains, all insects survived concentrations of 1000 ppm (Figure 1) representing at least 100,000-fold resistance to this product. A proportion of the individuals in all the populations collected was virtually immune to buprofezin. For example, the strain UK-1, which had never been exposed to buprofezin, had a similar $LC_{50}$ (0.012 ppm), to that of LAB-S. However, as shown in figure 2, resistant individuals within UK-1 caused a plateau in the dose-response curve from 1ppm upwards. Even 10,000 ppm would not kill these

resistant individuals. For teflubenzuron, the LAB-S strain was again very susceptible, with an $LC_{50}$ value of c. 2 ppm, but field collected strains showed high levels of survival and gave very flat plateaus when dose-response data were analysed. The possibility of cross-resistance between buprofezin and teflubenzuron, flagged by the similar patterns of resistance in field populations, was investigated by assessing resistance levels both before and after the selection

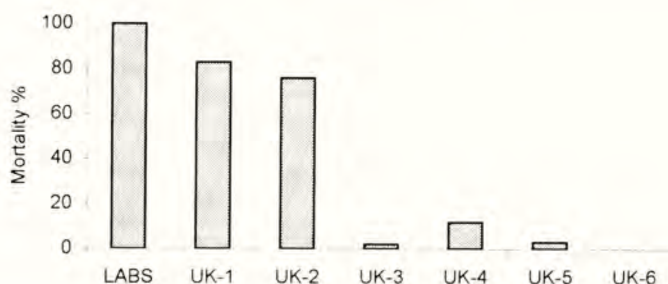
Table 1. Mortalities of glasshouse whitefly observed with original and selected colonies of UK-1 against buprofezin and teflubenzuron field rates.

| Treatment | Mortality in response to diagnostic dose (%)[1] | |
|---|---|---|
| | Buprofezin (75 ppm) | Teflubenzuron (75 ppm) |
| Control (no selection) | 71.1 | 68.1 |
| Buprofezin (75 ppm) | 16.4* | 26.2* |
| Teflubenzuron (75 ppm) | 80.7 | 72.6 |
| Teflubenzuron (1500 ppm) | 31.3* | 23.9* |

[1]Values followed by an asterisk are significantly different from their respective controls (Pearsons $\chi 2 > 65$, $p < 0.0001$).

of a colony with the appropriate compound. The UK-1 colony was chosen for its heterogeneous response to both insecticides, and this population was selected with the field-rate of both buprofezin and teflubenzuron (75 ppm). Selection of this strain with buprofezin also increased levels of resistance to teflubenzuron (Table 1). The same rate of teflubenzuron did not select resistance to either IGR, but increasing the selecting concentration to 1500 ppm teflubenzuron caused marked increases in resistance to both buprofezin and teflubenzuron.

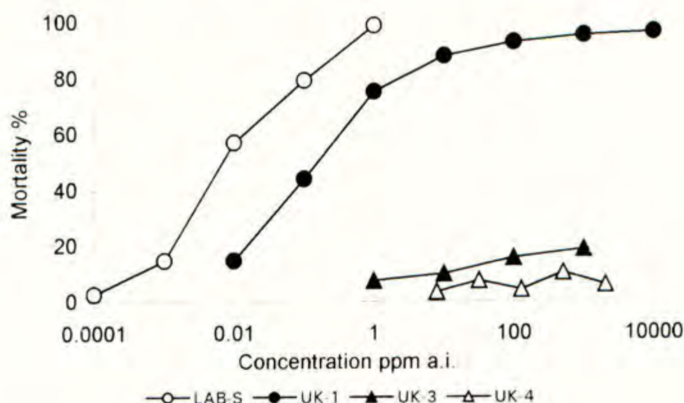
Figure 1. Percentage mortality of nymphs of a laboratory susceptible strain and six collections of *Trialeurodes vaporariorum* to a 1000 ppm concentration of buprofezin. The recommended field rate is 75 ppm, and the $LC_{50}$ value for a fully susceptible population is c. 0.01 ppm.




Imidacloprid

There is no evidence of imidacloprid resistance to date. The diagnostic dose of 128 ppm a.i., which kills 100% of susceptible insects, killed all individuals in UK populations. This situation was stable between 1997 and 1999, with repeat collections from the same sites exhibiting similar susceptibilities.

Figure 2. Response of representative UK strains of *Trialeurodes vaporariorum* to buprofezin




### *Tetranychus urticae*

Abamectin

The $LD_{50}$ for GSS was 0.03 ppm (slope = 2.7, 95% confidence limits = 0.02 - 0.04). There was no marked resistance in any of the UK strains, although TUK-1 did exhibit slightly (but significantly) increased tolerance. In this instance, the $LD_{50}$ was 0.09 ppm (slope = 2.7, 95% confidence limits = 0.06 - 0.11). This strain was from a cucumber glasshouse and the grower used fenbutatin oxide, not abamectin for control.

Fenbutatin oxide

The $LD_{50}$ for GSS was 9.7 ppm (slope = 1.8, 95% confidence limits = 6.0 - 14.0). Only one strain, TUK-2 exhibited marked resistance. In this instance, the $LD_{50}$ was 400 ppm (slope = 1.0, 95% confidence limits = 130 - 970). This strain was from an ornamental crop (lemon verbena) and fenbutatin oxide and abamectin had been used to control outbreaks of spider mite.

METI compounds

Initial assays, comparing the response of a range of UK strains to tebufenpyrad, showed that

Table 2. $LD_{50}$ values for *Tetranychus urticae* strains and the METI-acaricide, tebufenpyrad

| Strain | Number | $LC_{50}$ (95% CLs)[1] | Slope | RF[2] |
|---|---|---|---|---|
| GSS | 768 | 3.5 (2.3 - 4.9) | 2.0 | 1 |
| Akita | 357 | 225 ( 76 - 1180) | 1.3 | 65 |
| TUK-4 | 234 | 162 (74 - 306) | 1.1 | 46 |
| TUK-5 | 213 | 121 (77 - 194) | 1.3 | 35 |

[1]95% confidence limits around the mean. Non-overlapping values means $p < 0.05$.
[2]Resistance factor ($LD_{50}$ resistant / $LD_{50}$ susceptible)

most exhibited a similar response to GSS ($LD_{50}$ = 3.5 ppm) (Table 2). TUK-4, derived, not from a glasshouse, but from a hop-garden in Worcester showed increased tolerance ($LD_{50}$ = 162 ppm), and a very similar profile was shown by a single glasshouse collection from chrysanthemums (TUK-5; $LD_{50}$ = 121 ppm).

## DISCUSSION

Results of this survey show that resistance continues to be a primary force driving the use, choice and efficacy of pesticides in UK horticulture, and that this is an important constraint on the implementation of effective IPM strategies.

Laboratory bioassays, coupled with larger scale experiments in population cages ( K Gorman, unpublished data) support claims of the complete lack of control that many growers now report for buprofezin against *T. vaporariorum*. The effective loss of this compound, given its outstanding species selectivity and IPM compatibility, is likely to have important implications for the success of many existing control programmes and to hamper the transition of growers to biologically-based control of *T. vaporariorum*. Although it is often difficult to document treatment histories accurately, it appears that at least some of the sites now encountering severe resistance problems have not used this compound excessively. In our survey, insects capable of withstanding very high concentrations in bioassays were not confined to buprofezin-treated sites but also occurred in populations with no history of prior exposure to this chemical (UK-1 and one other from a cucumber grower who had never used buprofezin). One reason why resistance developed so quickly may be that resistant mutations might have been present at unexpectedly high frequencies even before the official release of buprofezin in the UK. This could have been caused by immigration from elsewhere in the world, or because the mutation that confers resistance was simply very common already. Whatever its causes, resistance to buprofezin is made more problematic by its stability. A laboratory strain maintained without selection for five years retains the same highly resistant profile as it did when it was collected, and we are aware of at least one instance of a grower who has not used buprofezin for three years but who still has a highly resistant whitefly population in his glasshouses (K Gorman, unpublished data).

The unexpected cross-resistance noted between buprofezin and teflubenzuron means that this latter compound must also be considered threatened. At present, teflubenzuron is licensed as suitable for integration into IPM programs for ornamentals. The fact that its efficacy is now also under debate further constrains the choice of IPM-friendly chemicals.

Imidacloprid remains particularly effective, but it is of concern that it is now being relied upon so heavily; often to the exclusion of all other chemicals. These factors act to increase the selection pressure for resistance to occur and we have already characterised imidacloprid resistance from another whitefly species, *Bemisia tabaci* (Cahill *et al.*, 1996b).

Of the compounds tested against *T. urticae* populations, fenbutatin oxide remains an effective acaricide for the majority of growers. Only one of our samples showed marked resistance, and this is in accord with the situation reported by Jacobson *et al.*, (1999) who found that, although resistance to this compound exists in some UK populations, such strains lost much of their resistance over time when they were maintained in the laboratory without further selection. In

the current study we also found abamectin to be very effective, with only one strain showing slight tolerance.

The strain TUK-4, collected in the autumn of 1999, although not from a glasshouse, was notable as the first for which control failures with METI-acaricides in Europe had been confirmed. Following this, a similar level of resistance was discovered in a glasshouse strain in April 2000 (TUK-5). This sample came from an ornamental crop geographically remote from the site where TUK-4 was collected. This suggests that METI-acaricide use in the UK is under threat. It is notable that resistance to tebufenpyrad affects the performance of all other compounds within this chemical class (Devine *et al.*, unpublished).

## ACKNOWLEDGEMENTS

The authors thank Ralf Nauen (Bayer) for providing the strain Akita, and Peter Taylor (Cyanamid UK) for bringing the field failure of tebufenpyrad to our attention. Work described here was supported by the Pesticide Safety Directorate of the UK Ministry of Agriculture, Fisheries and Food. IACR-Rothamsted receives grant-aided support form the Biological and Biosciences Research Council.

## REFERENCES

Cahill M; Jarvis W; Gorman K; Denholm I (1996a). Resolution of baseline responses and documentation of resistance to buprofezin in *Bemisia tabaci* (Homoptera: Aleyrodidae). *Bulletin of Entomological Research*, **86**, 117-122.

Cahill M; Gorman K; Day S; Denholm I (1996b). Baseline determination and detection of resistance to imidacloprid in *Bemisia tabaci* (Homoptera, Aleyrodidae). *Bulletin of Entomological Research*, **86**, 343-349.

De Cock A; Ishaaya I; Vandeveire M; Degheele D (1995). Response of buprofezin susceptible and buprofezin-resistant strains of *Trialeurodes vaporariorum* (Homoptera, Aleyrodidae) to pyriproxyfen and diafenthiuron. *Journal of Economic Entomology*, **88**, 763-767.

Dennehy T J; Farnham A W; Denholm I (1993). The microimmersion bioassay: a novel method for the topical application of pesticides to spider mites. *Pesticide Science*, **39**, 47-54.

Jacobson R J; Croft P; Fenlon J (1999). Response to fenbutatin oxide in populations of Tetranychus urticae Koch (Acari: Tetranychidae) in UK protected crops. *Crop Protection*, **18**, 47-52.

# Knock-down resistance (*kdr*) to pyrethroids in peach-potato aphids (*Myzus persicae*) in the UK: a cloud with a silver lining?

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## ABSTRACT

The recent discovery of a new mechanism, knock-down resistance (*kdr*), associated with strong resistance to pyrethroids in *M. persicae* has important implications for their future use for controlling this aphid pest. Diagnostic dose bioassays, coupled with molecular characterisation of *kdr* alleles, have shown *kdr* to be widespread in the UK and to be closely associated with overproduction of an insecticide-detoxifying carboxylesterase. As well as confering protection against pyrethroids, *kdr* appears to impair certain sensory functions in aphids that may account for a decline in the frequency of this resistance mechanism over recent years of less intensive insecticide application.

## INTRODUCTION

The peach-potato aphid, *Myzus persicae*, is a pest of major economic importance on a wide range of agricultural and horticultural crops throughout the world. Control measures still focus on insecticide application, which has selected for at least three different forms of resistance based on insecticide detoxification and altered target sites (Foster *et al.*, in press). Until 1990, only one resistance mechanism had been identified in *M. persicae*: the overproduction of one of two closely related carboxylesterases (E4/FE4) capable of sequestering and degrading insecticide esters (Devonshire & Moores, 1982). The MACE mechanism (**M**odified **A**cetyl**C**holin**E**sterase) was first detected in *M. persicae* in mainland Europe and the Far East (Moores *et al.*, 1994). This confers strong resistance specifically to the two dimethyl-carbamates, pirimicarb and triazamate. Finally, recent molecular-based studies have disclosed that some clones of *M. persicae* carry a point mutation leading to a single amino acid substitution (leucine to phenylalanine) in a voltage-gated sodium channel protein that is associated with strong resistance to deltamethrin and other pyrethroids (Martinez-Torres *et al.*, 1999). This alteration is also found in other pest species and is termed knock-down resistance or *kdr*.

## INSECTICIDE RESISTANCE IN *MYZUS PERSICAE* CONFERRED BY *KDR*

### Diagnosis

As well as conferring resistance to pyrethroid insecticides, *kdr*-type mechanisms provide cross-resistance to DDT, which also targets sodium channel proteins in the insect nervous system. Since pyrethroids can be resisted by mechanisms other than *kdr*, the most reliable and rapid test for this mechanism has been topical application of a single diagnostic dose of DDT to live aphids, followed by confirmatory molecular analysis of the sodium channel gene for the relevant point mutation using either the SSCP (Single Stranded Conformational

Polymorphism) technique (Williamson, *pers. comm.*) or DNA sequencing (Martinez-Torres *et al.*, 1999).

**Aphid bioassays**

Table I summarises the levels of resistance to DDT and the pyrethroid deltamethrin shown by *M. persicae* clones representing the three *kdr* genotypes. Aphids from each clone were exposed to topical applications of measured amounts of insecticide dissolved in acetone (Field *et al.*, 1997). *Kdr* genotypes were determined using the SSCP technique.

A clear distinction can be made between *M. persicae* clones homozygous (RR) or heterozygous (SR) for the point mutation associated with *kdr*, and ones lacking this mutation (SS), after exposure to DDT. The former group showed $ED_{50}$ values well over an order of magnitude greater than the latter. This pattern was repeated for deltamethrin, but with carboxylesterase (E4/FE4) levels also having a limited influence on resistance in *kdr*-SS clones. *Kdr* heterozygotes (SR) showed similar resistance to DDT and deltamethrin as *kdr* homozygotes (RR), with esterase resistance making some contribution to the RF values against the pyrethroid for both groups.

Table 1. Resistance factors for *Myzus persicae* clones assessed by topical bioassays using DDT and deltamethrin.

| clone | Resistance mechanism esterase[1] | *kdr*[2] | Resistance factor[3] DDT | deltamethrin |
|---|---|---|---|---|
| US1L | S | SS | **1.0**$^a$ | **1.0**$^a$ |
| 1238Z | S | SS | **1.6**$^a$ | **1.6**$^a$ |
| FrenchR | $R_2$ | SS | **1.4**$^a$ | **3.2**$^b$ |
| 800F | $R_3$ | SS | **0.7**$^a$ | **3.8**$^b$ |
| 1235Z | $R_2$ | SR | **64**$^b$ | **150**$^d$ |
| 1236Z | $R_3$ | SR | **92**$^b$ | **510**$^e$ |
| 923A | S | RR | **58**$^b$ | **35**$^c$ |
| 794J | $R_3$ | RR | **65**$^b$ | **540**$^e$ |

[1]S : susceptible; $R_2$: high-; $R_3$: extreme-resistance to organophosphates and monomethyl-carbamates
[2]*kdr* homozygote-susceptible: SS; *kdr* heterozygote: SR; *kdr* homozygote-resistant: RR
[3]Resistance factor = $ED_{50}$ for clone / $ED_{50}$ for US1L; values followed by the same letter do not differ significantly ($P < 0.05$) within each insecticide treatment

**Field simulators**

In addition to small scale aphid bioassays, resistance has also been evaluated for different clones of *M. persicae* supported on whole plants growing under semi-field conditions in field simulators (Foster & Devonshire, 1999) using aphicides applied at recommended field rates and volumes. Preliminary studies on potato (*Solanum tuberosum* cv Maris Piper) and Chinese

cabbage (*Brassica napus* var. *chinensis* cv. Tip-Top) have shown that the effectiveness of three commonly-used foliar pyrethroid sprays, lambda-cyhalothrin ('Hallmark'), deltamethrin ('Decis') and cypermethrin ('Toppel'), is highly dependent on whether *kdr* is present in the aphids (Foster, unpublished results). These products controlled non-*kdr M. persicae* relatively well, even when they carried extreme ($R_3$) esterase resistance. However, they were all ineffective against *M. persicae* with *kdr*. In contrast, pirimicarb ('Aphox') and a mixture of pirimicarb plus lambda-cyhalothrin ('Dovetail') were highly effective at controlling non-*kdr* and *kdr* aphids alike. Pirimicarb should therefore give good control of *M. persicae* on potatoes and brassicas in the absence of MACE resistance because it circumvents both the esterase and *kdr* mechanisms.

## INCIDENCE OF THE *KDR* MECHANISM IN *M. PERSICAE* ON UK CROPS

The occurrence of *kdr* in UK *M. persicae* samples collected primarily from field potatoes and brassicas, augmented by some glasshouse sites, was assessed between 1997 and 1999. Clones were isolated from each sample and replicates of 10 adult apterae from each clone were exposed to topical applications of diagnostic doses of DDT dissolved in acetone to establish their *kdr* phenotype (Martinez-Torres *et al.*, 1999). The data suggest that *kdr* is relatively widespread in *M. persicae* on UK crops (Figure 1). However, there appears to have been a downward trend in *kdr* frequency over the last three years (Figure 2A).

Taking the aphid samples that had insecticide treatment records, *kdr* was not only common in samples previously treated with pyrethroids but also in samples exposed only to organophosphates and carbamates (Table 2). This most probably resulted from tight association between *kdr* and esterase resistance in *M. persicae* in the UK, either due to close gene proximity or parthenogenesis that excludes genetic recombination. It appears therefore that *kdr* can be selected both directly by pyrethroids and indirectly through its association with other forms of resistance and *vice versa*. Indeed, high ($R_2$ and $R_3$) esterase resistance also appears to have declined over an equivalent period since 1996 (Figure 2B). Non-*kdr M. persicae* were nearly always restricted to aphid samples not recently treated with insecticides (Table 2).

Table 2. Total number of *Myzus persicae* clones showing *kdr* and non-*kdr* phenotypes isolated from 1997, 1998 and 1999 UK aphid samples that had insecticide treatment records.

| | No insecticides | | No pyrethroids | | Insecticides including pyrethroids | |
|---|---|---|---|---|---|---|
| | *kdr* | non-*kdr* | *kdr* | non-*kdr* | *kdr* | non-*kdr* |
| **1997** | 16 | 13 | 22 | 0 | 38 | 0 |
| **1998** | 5 | 8 | 0 | 2 | 7 | 0 |
| **1999** | 25 | 20 | 5 | 0 | 11 | 0 |
| | 46 | 41 | 27 | 2 | 56 | 0 |

*Kdr* genotypes were determined for a random fraction of the UK *M. persicae* clones isolated from the 1997 and 1998 samples (Table 3). All DDT-susceptible aphids (non-*kdr* phenotypes) were *kdr* homozygote-susceptible (SS). DDT-resistant aphids (*kdr* phenotypes) fell into two categories: *kdr* heterozygote (SR) or *kdr* homozygote-resistant (RR) with the former appearing to be the commonest genotype. DDT resistance was therefore always found in association with the *kdr* point mutation, confirming the utility of DDT bioassays as a routine diagnostic for the mechanism

Table 3. Association of *kdr* phenotypes (diagnosed by DDT bioassays) with *kdr* genotypes (determined by SSCP) in *M. persicae* clones isolated from aphid samples collected on UK crops in 1997 and 1998.

| | 1997 | | | 1998 | | |
|---|---|---|---|---|---|---|
| *kdr* genotype[1] | SS | SR | RR | SS | SR | RR |
| DDT-susceptible[2] | 5 | 0 | 0 | 11 | 0 | 0 |
| DDT-resistant[3] | 0 | 20 | 3 | 0 | 15 | 1 |

[1] *kdr* homozygote-susceptible: SS; *kdr* heterozygote: SR; *kdr* homozygote-resistant: RR.
[2] non-*kdr* phenotype [3] *kdr* phenotype

## POTENTIAL FITNESS COSTS ASSOCIATED WITH *KDR*

Laboratory studies in the absence of insecticides have provided strong evidence that *M. persicae* with *kdr* show much lower levels of response to synthetic aphid alarm pheromone, (*E*)-*β*-farnesene, than aphids lacking the *kdr* mechanism (Foster *et al.*, 1999). Furthermore, a recent winter field study has demonstrated that *kdr* homozygotes (RR) tend to remain longer on deteriorating excised leaves compared to aphids with the other *kdr* genotypes (Table 4). Both behaviours are potentially maladaptive because aphids may either be at greater risk of dying through predation, due to a reduced alarm response, or dying through starvation, due to becoming separated from their host plant after leaf senescence and subsequent abscission. These potential fitness costs are probably a direct pleiotropic effect of *kdr* itself as this resistance mechanism is due to a mutation in the voltage-gated sodium channel of nerve axon membranes: an alteration associated with reduced nerve activity (Vais *et al.*, 1997). Whatever the cause, the phenomenon most probably plays a crucial role in not only reducing the overall frequency of *kdr* forms in UK *M. persicae*, but also the frequency of other resistance mechanisms held in tight linkage disequilibrium with *kdr*.

## GENERAL DISCUSSION AND CONCLUSIONS

Contrary to previous belief, pyrethroid resistance in *M. persicae* appears to be based only to a limited extent on overproduction of detoxifying carboxylesterases (E4/FE4). Resistance appears to be primarily conferred by a mutant target site in the nervous system known as *kdr*. Initial selection for this mechanism probably arose from exposure to DDT in the 1950's and then persisted through cross-resistance to pyrethroids used since the 1970's. In the UK, *kdr* appears to

be closely associated with the production of elevated levels of E4 carboxylesterase. This linkage probably arose from the successive selection of clones by pyrethroid, carbamate and organophosphate insecticides and has probably been maintained by year-round parthenogenetic reproduction. Furthermore, prior to the discovery of *kdr*, the association between these two mechanisms promoted the false impression that high ($R_2$ and $R_3$) levels of esterase resistance conferred strong resistance to pyrethroids.

When *kdr* is present and MACE is absent, a situation that has prevailed over the last two years at most of the UK sites sampled, compounds unaffected by *kdr* including pirimicarb and pymetrozine should provide effective control and prove relatively benign to natural enemies. However, the efficacy of pirimicarb is constantly threatened by the reappearance of MACE, which was common in *M. persicae* on a range of crops in the Lincolnshire area in 1996 (Foster *et al.*, 1998).

Acknowledging that some sampling was biased towards crops with an aphid problem, there appears to be a prevalence of *M. persicae* in the UK showing *kdr* resistance. This suggests that the effectiveness of at least some pyrethroid applications made in the regions sampled could have been compromised. In the future it would be useful to assess *kdr* frequency in *M. persicae* collected in a more randomised manner such as in aphid suction traps (Woiwod & Harrington, 1994) to establish whether aphids on crops are representative of UK *M. persicae* as a whole. This approach is currently being used for monitoring esterase and MACE resistance in the UK. However, at present it is not possible to diagnose *kdr* by *in vivo* or *in vitro* tests on aphids caught in suction traps.

The apparent prevalence of *kdr* heterozygotes (SR) in UK *M. persicae* samples may reflect the fact that these forms show comparable high levels of resistance to DDT and pyrethroids as *kdr* homozygote-resistant (RR) aphids (Table 1) but respond more often to some important environmental cues for survival such as leaf deterioration. In other words, the SR forms appear to enjoy the benefit of resistance but apparently do not suffer quite as much from the associated fitness handicaps.

Table 4. Test of association between *kdr* resistance and proportion of *M. persicae* that remained on excised leaves in a winter field experiment (February 1999).

| | Mean proportions remaining | | | F value | df | P |
|---|---|---|---|---|---|---|
| *kdr* genotype[1] | SS | SR | RR | | | |
| | 0.42 | 0.45 | 0.68 | 6.17 | 2,20 | 0.008 |

[1]*kdr* homozygote-susceptible: SS; *kdr* heterozygote: SR; *kdr* homozygote-resistant: RR.

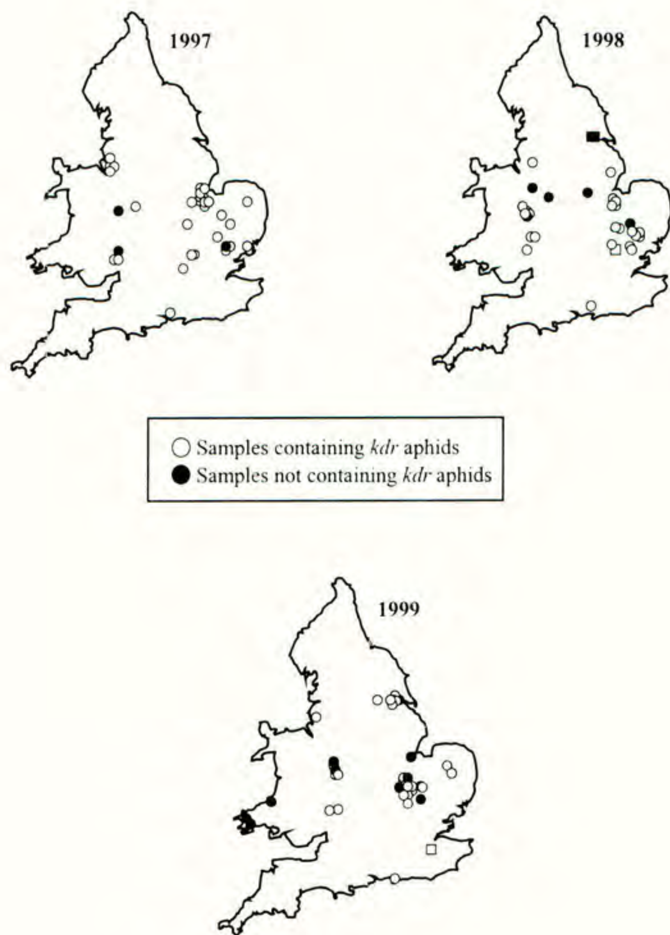


Figure 1. Incidence of *kdr* in *Myzus persicae* samples collected primarily from field crops (shown as circles) in England and Wales in 1997, 1998 and 1999. Samples collected from glasshouse crops are shown as squares.

The prevalence of parthenogenesis in *M. persicae* in the UK has important implications for the accumulation of different resistance mechanisms as it ensures that once they have been co-selected in the same aphid they will remain combined in clonal lineages for many subsequent generations. Hence, both the advantages and drawbacks conferred by one genetic factor, whether it is a resistance mechanism itself or an associated gene or gene complex, will affect any other mechanisms carried by that aphid clone. The stable close relationships built by parthenogenesis will therefore create non-independent fluctuations in the frequencies of the various forms of

resistance in UK populations of *M. persicae*. As a result, selection by an insecticide favouring one mechanism, will also benefit any associated mechanisms even if they do not confer resistance to that particular product. The reciprocal situation can also take place through adverse selection. Indeed, the propensity for *kdr* often to be found in MACE aphids and those with elevated levels of E4 carboxylesterase raises the possibility that these latter mechanisms can suffer adverse selection in field and glasshouse populations through potential fitness handicaps imposed by *kdr*. This hypothesis is supported by the observation that both MACE and esterase resistance have declined in frequency in the UK since their abnormally high levels in 1996. However, the possibility remains that these declines were partly influenced by reduced insecticide application coupled with immigration of susceptible clones from largely untreated crops or regions.

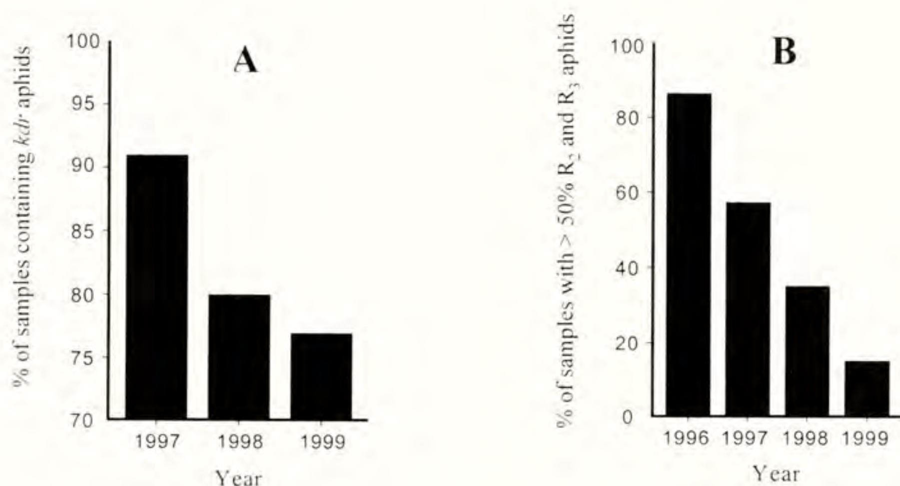


Figure 2. Percentage of *Myzus persicae* samples collected from UK crops that contained (**A**) aphids with *kdr* phenotypes and (**B**) greater than 50% aphids with high ($R_2$ and $R_3$) esterase resistance.

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## REFERENCES


Devonshire A L; Moores G D (1982). A carboxylesterase with broad substrate specificity causes organophosphorus, carbamate and pyrethroid resistance in peach-potato aphids (*Myzus persicae*). *Pesticide Biochemistry and Physiology* **18**, 235-246.

Field L M; Anderson A P; Denholm I; Foster S P; Harling Z K; Javed N; Martinez-Torres D; Moores G D; Williamson M S; Devonshire A L (1997). Use of biochemical and DNA diagnostics for characterising multiple mechanisms of insecticide resistance in the peach-potato aphid, *Myzus persicae* (Sulzer). *Pesticide Science* **51**, 283-289.

Foster S P; Denholm I.; Devonshire A L (2000). The ups and downs of insecticide resistance in peach-potato aphids (*Myzus persicae*) in the UK. *Crop Protection* (in press).

Foster S P; Denholm I; Harling Z K; Moores G D; Devonshire A L (1998). Intensification of insecticide resistance in UK field populations of the peach-potato aphid, *Myzus persicae* (Hemiptera: Aphididae) in 1996. *Bulletin of Entomological Research* **88**, 127-130.

Foster S P; Devonshire A L (1999). Field simulator study of insecticide resistance conferred by esterase-, MACE- and *kdr*-based mechanisms in the peach-potato aphid, *Myzus persicae* (Sulzer). Pesticide Science **55**, 810-814.

Foster S P; Harrington R; Devonshire A L; Denholm I; Devine G J; Kenward M G; Bale J S (1996). Comparative survival of insecticide-susceptible and resistant peach-potato aphids, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), in low temperature field trials. *Bulletin of Entomological Research* **86**, 17-27.

Foster S P; Woodcock C M; Williamson M S; Devonshire A L; Denholm I; Thompson R (1999). Reduced alarm response for peach-potato aphids (*Myzus persicae*) with knock-down resistance to insecticides (*kdr*) may impose a fitness cost through increased vulnerability to natural enemies. *Bulletin of Entomological Research* **89**, 133-138.

Martinez-Torres D; Foster S P; Field L M; Devonshire A L; Williamson M S (1999). A sodium channel point mutation is associated with resistance to DDT and pyrethroid insecticides in the peach-potato aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae). *Insect Molecular Biology* **8**, 1-8.

Moores G D; Devine G J; Devonshire A L (1994). Insecticide-insensitive acetylcholinesterase can enhance esterase-based resistance in *Myzus persicae* and *Myzus nicotianae*. *Pesticide Biochemistry and Physiology* **49**, 114-120.

Vais H; Williamson M S; Hick C A; Eldursi N; Devonshire A L; Usherwood P N R (1997). Functional analysis of a rat sodium channel carrying a mutation for insect knock-down resistance (*kdr*) to pyrethroids. *Federation of European Biochemical Letters* **413**, 327-332.

Woiwod I P; Harrington R (1994). Flying in the face of change: the Rothamsted insect survey. In: *Long-term experiments in agricultural and ecological sciences*, eds R A Leigh & A E Johnston, pp 321-342. CAB International, Wallingford.