POSTER SESSION 6C

FUNGICIDE AND INSECTICIDE RESISTANCE – CURRENT STATUS AND FUTURE MANAGEMENT

Session Organiser: Dr D D Slawson
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Poster Papers: 6C-1 to 6C-9

Confirmation of insecticide resistance in UK populations of the currant-lettuce aphid, *Nasonovia ribisnigri*

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ABSTRACT

Bioassay data for a reference strain of the currant-lettuce aphid, *Nasonovia ribisnigri*, were used to assess insecticide resistance in field strains of this species from outdoor lettuce in the UK. Results showed widespread but varied levels of resistance to pirimicarb (carbamate) and cypermethrin (pyrethroid) but only low resistance to heptenophos (organophosphate). There was no biochemical evidence of an altered acetylcholinesterase contributing to pirimicarb resistance in any of the strains tested.

INTRODUCTION

In the UK, lettuce foliage is colonised by several aphid species including the currant-lettuce aphid, *Nasonovia ribisnigri*, the peach-potato aphid, *Myzus persicae*, and the potato aphid, *Macrosiphum euphorbiae*. *N. ribisnigri* is often the major pest and is more specific to lettuce than the other two species. Insecticide resistance is widespread and well documented in *M. persicae* (e.g. Foster *et al.*, 1998) but has so far only been demonstrated in *N. ribisnigri* in southern Europe (Rufingier *et al.*, 1997).

This study was initiated following reports of insecticides becoming less effective against *N. ribisnigri* in the UK. Bioassays conducted with several compounds against two strains of *N. ribisnigri*, including one strain considered to exhibit baseline susceptibility to insecticides, are reported. Nine further strains recently collected from the field were tested with cypermethrin, heptenophos and pirimicarb. Comparisons of acetylcholinesterase (AChE, the target site of organophosphates and carbamates) kinetics were conducted to investigate the possible role of this enzyme in conferring resistance.

MATERIALS AND METHODS

Aphid strains and rearing methods

The two strains tested in most detail were: ROTH, a susceptible laboratory strain initially established at HRI Wellesbourne in the summer of 1994 and transferred to Rothamsted in 1995, and 1312, collected in Kent in 1997 from lettuce crops on which control with pirimicarb had not been effective. Nine field strains collected during 1997 from northern (3

strains), central (1 strain), south-eastern (3 strains) and eastern (2 strains) England were tested in less detail and discarded within three generations of receipt.

All strains of *N. ribisnigri* were reared parthenogenetically in the laboratory, without cloning or exposure to insecticides, on whole plants of lettuce (*Lactuca sativa* cv. 'Webb's Wonderful' or 'Iceberg') at 21°C with a 16:8h (L:D) photoperiod. Plants were changed regularly to avoid excessive crowding of aphids.

Insecticides

Formulated insecticides used for leaf-dip bioassays were cypermethrin ('Polytrin', 200g/l EC); deltamethrin ('Decisquick', 25g/l EC); lambda-cyhalothrin ('Hallmark', 50g/l EC); heptenophos ('Hostaquick', 550g/l EC); demeton-S-methyl ('Metasystox 55', 580g/l EC); dimethoate ('Danadim', 400g/l EC) and pirimicarb ('Aphox', 500g/kg soluble granules). For leaf-dipping, all formulations were diluted to the required concentration in distilled water containing 0.01% of the non-ionic surfactant 'Agral' (Zeneca Agrochemicals).

Bioassays

Leaf discs (35 mm diameter) cut from lettuce (cv. 'Webb's Wonderful' or 'Iceberg') were dipped in insecticide solution for 20 s, placed upside down on an agar bed (25 mm in depth) in disposable plastic containers (30 mm high), and allowed to air-dry. Alate adults of the required strain (10 per container) were placed on the treated leaf surface. Leaf discs dipped in water plus 'Agral' were used as controls. Bioassays were stored for 72 h in a constant environment facility at 20°C under ambient daylight conditions. Adults incapable of co-ordinated movement of legs were scored as dead.

Dose-response bioassays against ROTH and 1312 used three batches of 10 aphids (*i.e.* 30 insects) at at least five insecticide concentrations. Each bioassay was replicated at least twice with results pooled for probit analysis using the POLO computer program (LeOra Software, Berkeley, California). Resistance factors for the 1312 strain were calculated by dividing its fitted LC_{50} values by the corresponding LC_{50} for the ROTH strain. Other field strains were tested only with cypermethrin, heptenophos and pirimicarb, usually on just one occasion with 5-6 concentrations and two batches of 10 alate adults per concentration. Owing to this low number of insects and the possibility of genetic heterogeneity within strains, no attempt was made to fit probit lines to these data.

Characterisation of AChE

Homogenates of approximately 60 aphids in 750µl of 0.1M phosphate buffer (pH 7.5 containing 0.1% Triton X-100), were centrifuged at 1100g for 30 s and the supernatant taken. AChE activity was assayed at 25°C using a Thermomax microplate reader (Molecular Devices). Inhibition was measured in the absence of substrate by mixing 100µl enzyme with known concentrations of pirimicarb and assaying the remaining activity at 90 s intervals over 5 min by mixing 20µl of the homogenate with 100µl acetylthiocholine iodide (ATChI) and 50µl dithiobis-(2-nitrobenzoic acid) (DTNB) to give final concentrations of 0.5mM and 15µM respectively. Bimolecular rate constants (k_i) were calculated by non-linear regression using Grafit software (Leatherbarrow). Michaelis Constant (K_m) values were determined by

non-linear regression of enzyme activity measurements over 2 min with 12 concentrations of ATChI ranging from 0.05 to 10 mM.

RESULTS

Bioassays against standard strains and clones

Compared to ROTH, 1312 exhibited little or no resistance to the pyrethroids cypermethrin, deltamethrin and lambda-cyhalothrin. LC_{50}s of 1312 to the organophosphates (OPs) heptenophos and demeton-S-methyl were significantly higher (4- to 5-fold) than those for ROTH, whereas responses to dimethoate did not differ significantly. The highest resistance recorded in 1312 was to pirimicarb (11-fold) (Table 1).

Table 1. Probit analysis of responses of three strains of *Nasonovia ribisnigri* to insecticides.

	ROTH			1312			
Insecticide	LC_{50}[1]	CL[2]	Slope	LC_{50}[1]	CL[2]	Slope	RF[3]
Cypermethrin	2.1	1.7 - 2.5	4.1	5.4	4.2 - 6.6	2.0	2.6
Deltamethrin	0.29	0.18 - 0.39	3.4	0.48	0.25 - 0.75	1.9	1.7
Lambda-cyhalothrin	0.096	0.058 - 0.13	4.0	0.15	0.098 - 0.20	2.7	1.6
Heptenophos	24	18 - 29	4.9	110	90 - 130	5.3	4.6
Demeton-S-Methyl	6.7	4.7 - 8.9	1.9	30	20 - 41	2.2	4.5
Dimethoate	5.5	4.1 - 7.1	3.0	13	4 - 20	3.2	2.4
Pirimicarb	5.9	4.2 - 7.9	2.0	62	46 - 81	2.6	11

1. Expressed as ppm a.i.
2. 95% confidence limits for fitted LC_{50} values.
3. RF = resistance factor

Response of field strains to insecticides

Since data for other field strains were not subjected to probit analysis, dose-mortality data for each are plotted in Figure 1 as observed means alongside pooled results from repeated bioassays against the ROTH strain. With cypermethrin (Figure 1a), field strains varied from being fully susceptible to exhibiting less than 20% mortality at 20 ppm (equivalent to at least 10-fold resistance). In the most resistant strains, 10% of insects survived a concentration of 80 ppm, *c.* 40-fold higher than the LC_{50} of ROTH aphids. When tested with heptenophos (Table 1b), several field strains responded similarly to ROTH, whereas others apparently conformed to the *c.* 4-fold resistance documented for 1312. All nine field strains were more tolerant of pirimicarb than ROTH (Figure 1c), but the magnitude of resistance varied considerably between strains. At a concentration of 50 ppm pirimicarb (corresponding approximately to LC_{50} values of 1312, and which consistently killed all ROTH insects), mortality of field strains ranged from 22% to 100%.

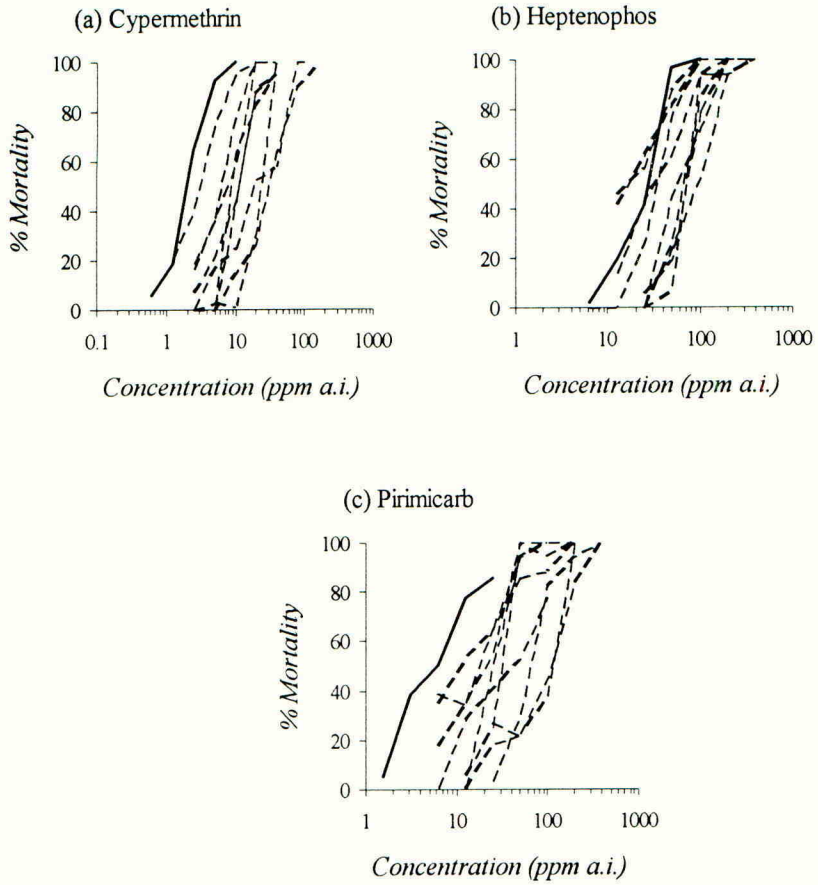

Figure 1. Response of nine field strains of *Nasonovia ribisnigri* (shown as dashed lines) to three insecticides. Solid lines show base-line responses of the susceptible ROTH strain.

Characterisation of AChE

K_m values expressing AChE affinity for the model substrate acetylthiocholine iodide did not differ significantly between the ROTH and 1312 strains (Table 2) and k_i values were also similar. Hence there was no evidence of qualitative differences in AChE between strains and no basis for implicating target-site modification as the cause of resistance to pirimicarb in 1312. AChE assays against field strains using a single discriminating concentration of pirimicarb showed no differences in AChE sensitivity to the chemical.

Table 2. Michaelis Constant (K_m, mM), and bimolecular rate constant (k_i, M^{-1} min^{-1} ± SE) for inhibition of *Nasonovia ribisnigri* AChE by pirimicarb.

Strain	K_m	k_i
ROTH	0.34 ± 0.071	$3.8 \pm 0.9 \times 10^5$
1312	0.24 ± 0.066	$2.8 \pm 1 \times 10^5$

DISCUSSION

Characterisation of insecticide resistance in aphids on lettuce is complicated by the frequent occurrence of more than one species, and the difficulties encountered by growers in identifying these species. Some reports of control failures are likely to relate to *M. persicae*, in which multiple resistance mechanisms have been documented and proved capable of impairing the field performance of several insecticides (e.g. Field *et al.*, 1997; Foster *et al.*, 1998). However, it would be naive to ascribe all difficulties in achieving control to *M. persicae* since *N. ribisnigri*, with its very restricted host range, is potentially very vulnerable to selection for resistance. This has already been confirmed in populations of *N. ribisnigri* from areas of intensive lettuce production in south-western Europe (Rufingier *et al.*, 1997).

Repeated bioassays against the strain 1312, suspected of resisting pirimicarb, showed consistent response patterns with resistance being highest (11-fold at LC_{50}) to this insecticide. Although this resistance factor appears somewhat low to account for difficulties with control, relationships between laboratory bioassay data and the field performance of insecticides can be complex (e.g. Sawicki, 1987). Further research is needed to investigate these relationships for *N. ribisnigri*. In addition, it is conceivable that 1312 had undergone a decline in pirimicarb resistance since being collected from the field. 1312 exhibited lower but significant resistance to the organophosphate heptenophos and demeton-S-methyl, and no apparent resistance to the organophosphate dimethoate or to pyrethroids.

Responses of nine further field strains of *N. ribisnigri* to pirimicarb varied considerably, and although resistance factors were not quantified for these strains, results appeared consistent with the 4- to 19-fold range of resistance reported at LC_{50} for four strains from south-western Europe (Rufingier *et al.*, 1997). Some of these field strains exhibited greater tolerance of cypermethrin than 1312, implying a potential for significant resistance to pyrethroids that needs to be verified through selection experiments and/or more detailed monitoring at sites where pyrethroids are used most intensively. Among the nine field strains examined, there was no clear correlation between responses to cypermethrin and pirimicarb; some strains displaying the greatest tolerance of cypermethrin being among the most susceptible to pirimicarb, and *vice versa*.

So far there is no evidence for an insecticide-insensitive variant of AChE in *N. ribisnigri*, although this mechanism can be anticipated to occur given its importance in enhancing resistance to pirimicarb in both *M. persicae* (Moores *et al.*, 1994) and *A. gossypii* (Moores *et*

al., 1996 and references therein). Further research is required to resolve the mechanism(s) of resistance in *N. ribisnigri*.

Confirmation of seemingly widespread resistance in UK populations of *N. ribisnigri* highlights the importance of future work to characterise in more detail its distribution and underlying mechanisms. The co-existence of two species of aphid resistant to insecticides (*N. ribisnigri* and *M. persicae*) on such a high value crop as lettuce is of great concern and should promote increased attention to less chemically-dependent control strategies.

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REFERENCES

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

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.

Moores G D; Gao X W; Denholm I; Devonshire A L (1996). Characterisation of insensitive acetylcholinesterase in insecticide-resistant cotton aphids, *Aphis gossypii*. *Pesticide Biochemistry and Physiology* **56**, 102-110.

Rufingier C; Schoen L; Martin C; Pasteur N (1997). Resistance of *Nasonovia ribisnigri* (Homoptera: Aphididae) to five insecticides. *Journal of Economic Entomology* **90**, 1445-1449.

Sawicki R M (1987). Definition, detection and documentation of insecticide resistance. In: *Combating Resistance to Xenobiotics*, eds M G Ford; D W Hollomon; B P S Khambay; R M Sawicki, Ellis Horwood: Chichester.

Response of European populations of the glasshouse whitefly, *Trialeurodes vaporariorum*, to conventional and novel insecticides

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ABSTRACT

Although insecticide resistance has long been recognised in the glasshouse whitefly, *Trialeurodes vaporariorum,* there is a shortage of recent data on the incidence and breadth of resistance in this species, especially in relation to newer compounds (e.g. imidacloprid and buprofezin) that are well-suited for incorporation into IPM programmes. To address this shortcoming, samples of *T. vaporariorum* were collected from four sites in the UK and from Germany, Spain and the Netherlands, and tested for resistance to bifenthrin (a pyrethroid), profenofos (an organophosphate) as well as imidacloprid and buprofezin. Results were compared to those for a strain (LAB-S) that had been reared for at least 25 years without exposure to insecticides. With bifenthrin and profenofos, some strains survived very high concentrations but others proved as susceptible as LAB-S. Responses to buprofezin also varied considerably and some strains proved effectively immune to this chemical. In contrast, none of the strains showed evidence of resistance to imidacloprid.

INTRODUCTION

The glasshouse whitefly, *Trialeurodes vaporariorum* is a serious pest of horticultural crops in many parts of the world. In some glasshouse and greenhouse systems, especially those producing food crops, control of *T. vaporariorum* has switched very largely from the use of insecticides to inundative or inoculative releases of the Aphelinid parasitoid, *Encarsia formosa* (e.g. van Lenteren, 1985), which is available from several commercial sources. In others, insecticides retain an important role, although there is increasing emphasis on chemicals considered most benign to natural enemies, in order to promote the implementation of IPM for whiteflies and co-existing pest species.

The first detailed studies of insecticide resistance in *T. vaporariorum* were undertaken in the early 1970s by L.R. Wardlow and co-workers in the UK (Wardlow *et al.,* 1972, 1975, 1976). This work identified substantial resistance to organophosphates (OPs) as well as increasing problems with resistance to newly-introduced pyrethroid insecticides. Since then there has been little attention paid in Europe to monitoring the susceptibility of *T. vaporariorum* to conventional insecticides or to newer agents introduced for whitefly control including the insect growth regulator, buprofezin (Wilson & Anema, 1988), and the chloronicotinyl, imidacloprid (Oetting & Anderson, 1990). To address this shortcoming, the resistance status of contemporary populations of *T. vaporariorum* in the UK and elsewhere to a range of chemicals, and its implications for managing whiteflies in protected horticulture were investigated. Some results of this research are reported here.

MATERIALS AND METHODS

Insect strains

Field strains of *T. vaporariorum* were collected from four counties in England (Essex, Cambridge, Worcester and Somerset), and from single localities in Spain, Germany and the Netherlands (Table 1). The Essex strain had been used for c. 15 years by a biocontrol company for commercial rearing of *Encarsia formosa*, and had not knowingly been exposed to insecticides during this period. All other field strains came from commercial plant production glasshouses or greenhouses with varied (and sometimes poorly documented) spray histories. Results for field strains were compared with those for a long-standing laboratory strain (LAB-S) originally established by L.S Wardlow in the UK in the early 1970's, but maintained for the last ten years in an unsprayed glasshouse at Cornell University, Ithaca, USA (J. P. Sanderson, pers. comm.). At Rothamsted, all colonies were reared on dwarf French bean plants (cv. 'Canadian Wonder') under a 16 h photoperiod at 22°C, and maintained without exposure to insecticides. Adult insects used in bioassays were aged between 2 and 8 days old.

Table 1. Origin of field strains of *T.vaporariorum*.

Strain	Year of collection	Origin	Host plant
UK-1	1994	Essex	French bean
UK-2	1997	Cambridge	*Hibiscus* spp.
UK-3	1997	Worcester	Tomato
UK-4	1997	Somerset	*Fuchsia* spp.
SPA	1994	Spain	Tomato
GER	1995	Germany	Cucumber
NED	1995	Netherlands	Aubergine

Insecticides

Insecticides were applied as formulated products as follows: bifenthrin ('Capture', 25% EC); profenofos, ('Curacron', 50% EC); imidacloprid, ('Confidor', 25% EC); and buprofezin, ('Applaud', 25% EC). All were diluted to required concentrations with water containing 0.01% of the non-ionic wetter 'Agral'.

Bioassays

Leaf-dip bioassays with bifenthrin and profenofos against adults were based on Cahill *et al.* (1995). Females were confined to discs (37 mm diameter) cut from dwarf French bean leaves laid on an agar bed inside ventilated plastic Petri-dishes (38 mm diameter, 20 mm high). The leaf discs had previously been dipped into insecticide solution for 20 s and allowed to air-dry. Controls were treated in the same way except that discs were dipped into the diluent only. Leaf-dip bioassays consisted of three replicates of 20-30 insects per concentration, and were maintained at 22°C with final mortality scored 48 h after initial exposure.

Imidacloprid was tested using a systemic uptake bioassay following Cahill *et al.* (1996a). Leaves were cut from bean plants and the petiole immersed for 40 h into the required

Petri-dishes as for the leaf-dip test (see above). Leaves immersed in distilled water were used for controls. Bioassays consisted of three replicates of 20-30 adult females per concentration, held at 22°C, with adult mortality scored after 72 h.

Buprofezin was tested against early instar whitefly nymphs following Cahill *et al.* (1996b). Leaves on bean plants were trimmed into rectangles of approximately 50 mm x 40 mm. Adult females were confined to these in clip cages for 20-24 h, to provide a cohort of eggs in a small area that was easily assessed. 10-12 days later, infested leaves were dipped into solutions of buprofezin. Water plus 'Agral' was used for controls. Mortality was assessed 22-25 days after egg laying, when survivors had reached the fourth instar stage and dead nymphs were easily distinguished. Bioassays consisted of 3-4 replicates per concentration.

Analysis of data

When appropriate, dose-response data were subjected to probit analysis using the POLO computer program (LeOra Software, Berkeley, California), and resistance factors calculated by dividing fitted LC_{50}s by those for LAB-S whiteflies. In some cases, mortality was too low or too heterogeneous over the concentrations tested to permit probit lines to be fitted.

RESULTS AND DISCUSSION

Dose-response data for all strains are plotted in Figures 1 and 2 as mean (untransformed) percentage mortality at increasing concentrations of insecticide. LC_{50}s and resistance factors, when calculable, are listed in Table 2 for all four insecticides. Due to the scarcity of comparable data for *T. vaporariorum* in the literature, interpretation of results is assisted by reference to baseline data for the tobacco whitefly, *Bemisia tabaci*, against which all four insecticides have been tested extensively using similar bioassay methods (e.g. Cahill *et al.*, 1995, 1996a, 1996b). LC_{50} values for a fully susceptible strain (SUDS) of *B. tabaci* are also listed in Table 2.

Responses of three field strains (UK-1, GER and NED) to bifenthrin were very similar to that of the LAB-S strain (Figure 1a), showing linear dose-response relationships and LC_{50} values in the range 1.9-3.7ppm a.i. (Table 2). Although 6- to 9-fold higher than the corresponding LC_{50} for susceptible *B. tabaci*, also calculated after 48 h exposure, these figures appear to represent baseline susceptibility of *T. vaporariorum* to bifenthrin. Responses of the other four strains were displaced to the right of the concentration axis, showing a maximum of 50% mortality at 100 ppm. This signifies at least 20-fold resistance relative to LAB-S (Table 2). The disparity between UK-I on the one hand, and UK-2, UK-3, and UK-4 on the other, was consistent with differences in their reported treatment histories; UK-1 having been maintained for many years without insecticide exposure, but the other three having been subjected to commercial pest management regimes.

Responses to profenofos followed a similar pattern to those for bifenthrin, with four field strains resembling LAB-S and three showing at least 10-fold greater tolerance (Figure 1b). In this case, the range of 'baseline' LC_{50}s (160-340) was at least 25-fold higher than that for susceptible *B. tabaci* (Table 2). While this could reflect a major difference in intrinsic toxicity to the two species, it may also indicate the ubiquitous presence, even in LAB-S, of a basal mechanism of resistance to profenofos and other OPs. Biochemical comparisons of

to the two species, it may also indicate the ubiquitous presence, even in LAB-S, of a basal mechanism of resistance to profenofos and other OPs. Biochemical comparisons of acetylcholinesterase enzymes in *T. vaporariorum* and *B. tabaci* (G. Moores, pers. comm.) support this hypothesis, which is being investigated further. If correct, the higher resistance mode evident for strains such as UK-4 would signify the occurrence in some populations of a second, possibly metabolic mechanism of OP resistance.

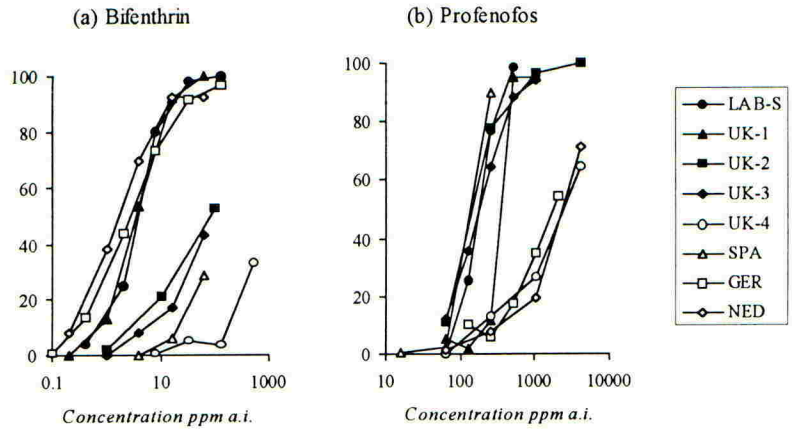

Figure 1. Dose-response relationships disclosed by testing eight strains of *T. vaporariorum* with (a) befenthrin and (b) profenofos.

Table 2. LC_{50} values (ppm a.i.) and resistance factors (RF) from bioassays with eight strains of *T. vaporariorum*.

	Bifenthrin		Profenofos		Imidacloprid		Buprofezin	
Strain	LC_{50}	RF	LC_{50}	RF	LC_{50}	RF	LC_{50}	RF
LAB-S	3.7	1.0	180	1.0	5.2	1.0	0.010	1.0
UK-1	3.5	0.95	340	1.9	3.0	0.58	0.12	12
UK-2	78	21	160	0.89	6.8	1.4	nc	-
UK-3	87	24	180	1.0	4.1	0.79	nc	-
UK-4	nc	-	2400	13	nt	-	nc	-
SPA	nc	-	160	0.89	2.8	0.54	2.5	250
GER	2.9	0.78	nc	-	9.3	1.8	5.2	520
NED	1.9	0.51	2300	13	8.0	1.6	nt	-
SUDS*	0.34		6.1		1.4		0.27	

nc = LC_{50} values not calculable due to insufficient mortality in bioassays.
nt = not tested. * Comparable data for a susceptible strain of *B. tabaci*.

LC_{50} values for imidacloprid ranged from 2.8 to 9.3 ppm, compared with a value of 1.4 ppm from bioassays against susceptible *B. tabaci* (Table 2). All strains responded very similarly and showed no evidence of resistance to this compound (Figure 2a). To our knowledge, the only

LAB-S whiteflies proved extremely susceptible to buprofezin. Their LC_{50} (0.01 ppm) was substantially lower than that for susceptible *B. tabaci* (0.27 ppm; Table 2), and values from comparable bioassays against susceptible strains of *T. vaporariorum* from the Netherlands (2.1 ppm, De Cock *et al.*, 1995) and New Zealand (0.14 ppm, Workman & Martin, 1995). Although the LC_{50} for UK-1 (0.012 ppm) was closer to other baseline estimates, this strain surprisingly exhibited a 'plateau' with c. 5% of insects surviving very high concentrations of buprofezin (Figure 2b). This has since been confirmed to represent a low frequency of heritable resistance rather than an artifact of the bioassay (unpublished data). Reasons for its occurrence in a population with no history of prior exposure to buprofezin are being investigated further. It is notable, however, that the other three UK strains showed even clearer evidence of resistance, with two (UK-3 and UK-4), both from sites reporting control failure with buprofezin, being largely unaffected by the highest concentrations applied (up to 5000 ppm). Strong resistance of *T. vaporariorum* has previously been reported from the Netherlands (De Cock *et al.*, 1995) and New Zealand (Workman & Martin, 1995), and now appears to be becoming established in the UK.

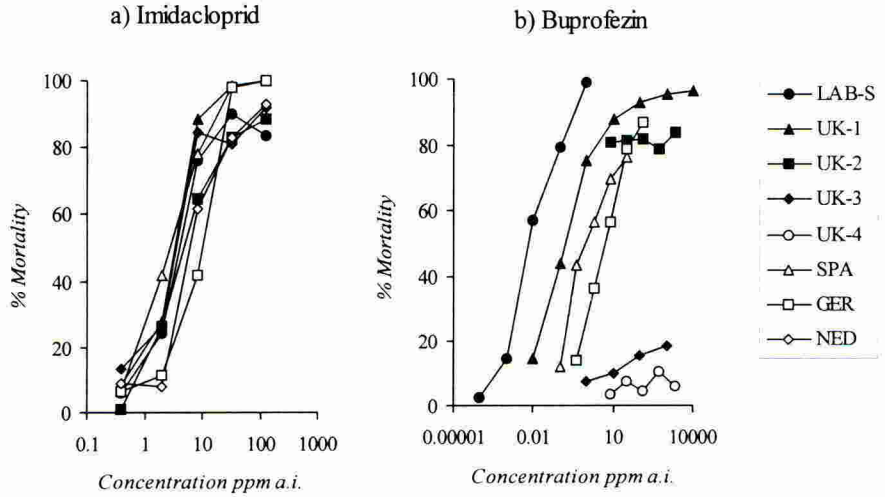

Figure 2. Dose-response relationships disclosed by testing eight strains of *T. vaporariorum* with (a) imidacloprid and (b) buprofezin.

In summary, there is clearly still considerable variation between populations for resistance to older insecticides such as bifenthrin and some OPs, which could retain a role in whitefly control at sites where side-effects on beneficial species are not a prime consideration. The most alarming finding is the magnitude of resistance in some UK populations to buprofezin, just 3-4 years after its commercial release in this country. This highlights the urgent need to moderate use of novel insecticides to combat intrinsic genetic and ecological factors intensifying selection for resistance in protected environments (e.g. Denholm *et al.*, 1998).

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REFERENCES

Cahill M; Byrne F J; Gorman K; Denholm I; Devonshire A L (1995). Pyrethroid and organophosphate resistance in the tobacco whitefly, *Bemisia tabaci* (Homoptera: Aleyrodidae). *Bulletin of Entomological Research* **85**, 181-187.

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

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

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

Denholm I; Cahill M; Dennehy T J; Horowitz A R (1998). Challenges with managing insecticide resistance in agricultural pests, exemplified by the whitefly, *Bemisia tabaci*. *Philosophical Transactions of the Royal Society, Series B* (in press).

van Lenteren J C (1985). Parasitoids in the greenhouse: successes with seasonal inoculative release systems. In: *Insect parasitoids*, eds. J Waage & D Greathead, pp. 341-374. Academic Press: London.

Oetting R D; Anderson A L (1990). Imidacloprid for control of whiteflies, *Trialeurodes vaporariorum* and *Bemisia tabaci*, on greenhouse grown poinsettias. *Proceedings 1990 Brighton Crop Protection Conference* - Pests and Diseases - 1990 **1**, 367-372.

Wardlow L R; Ludlam F A B; French N (1972). Insecticide resistance in glasshouse whitefly. *Nature* **239**, 164-165.

Wardlow L R; Ludlam F A B; Hammon R P (1975). A comparison of the effectiveness of insecticides against glasshouse whitefly (*Trialeurodes vaporariorum*). *Annals of Applied Biology* **81**, 433-435.

Wardlow L R; Ludlam A B; Bradley L F (1976). Pesticide resistance in glasshouse whitefly (*Trialeurodes vaporariorum* West.). *Pesticide Science* **7**, 320-324.

Wilson D; Anema B P (1988). Development of buprofezin for control of whitefly *Trialeurodes vaporariorum* and *Bemisia tabaci* on glasshouse crops in The Netherlands and the UK. *Proceedings Brighton Crop Protection Conference - Pests and Diseases - 1988* **1**, 175-180.

Workman P J; Martin N A (1995). Greenhouse whitefly (*Trialeurodes vaporariorum* (Westwood), Hemiptera: Aleyrodidae) resistance to buprofezin. *Proceedings 48th New Zealand Plant Protection Conference*, 31-34.

Resistance in *Myzus persicae*: Current status in Europe and future prospects

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ABSTRACT

Populations of the peach-potato aphid, *Myzus persicae* were sampled from field and glasshouse crops from 180 sites in Europe, and their resistance classified as S, R_1, R_2, or R_3 according to esterase activity measured by immunoassay, and as having normal or modified (MACE) acetylcholinesterase as judged by the enzyme's sensitivity to pirimicarb. Esterase-based resistance predominated in most samples, although there was considerable variation in the proportions of the different esterase levels both within and between countries. Likewise, MACE was found in England, Holland, Greece and Italy, but again with marked differences between sampling sites, reflecting different selection pressures and/or migration patterns. The implications for controlling *M. persicae* are discussed.

INTRODUCTION

The most ubiquitous resistance mechanism in *Myzus persicae* is that of elevated esterase (E4/FE4) which causes resistance to organophosphorus (OP) and carbamate compounds (Devonshire & Moores, 1982). This mechanism also confers some resistance to pyrethroids but the major contribution is from a target site mechanism, knockdown resistance (*kdr*), which has recently been shown to be tightly linked with the E4 mechanism, and the two appear to have been co-selected over many years (Martinez-Torres *et al.*, 1997). Another target site mechanism, modified acetylcholinesterase (MACE), has recently become important in some regions (Moores *et al.*, 1994a and b), in conjunction (though not linked) with the elevated esterase mechanism. MACE confers resistance to the dimethylcarbamate compounds pirimicarb and triazamate, which can provide good control of aphids with esterase- or *kdr*- based resistance whilst also offering a selective aphicidal alternative to broader spectrum compounds.

This paper summarises the results of a survey of European *M. persicae* populations sampled by staff of Zeneca and ADAS in 1997, and analysed at Rothamsted for the esterase and MACE mechanisms. The implications for controlling aphids with these mechanisms are discussed.

METHODS

Aphids were sampled from field and glasshouse crops in the UK, Germany, Greece, Holland and Italy. In all, 4,760 aphids were analysed from 180 sites, an average of 26 per site. Each was classified as S, R_1, R_2, or R_3 according to esterase activity measured by immunoassay (Devonshire *et al.*, 1986), and as having normal or modified (MACE) acetylcholinesterase as judged by the enzyme's sensitivity to pirimicarb (Moores *et al.*, 1994a).

RESULTS

UK

Esterase-based resistance has long been recognised in UK populations of *M. persicae*, and its incidence in different crops has been monitored over the last 20 years. The proportion of resistant variants has shown little systematic variation between the 1980s and the mid-1990s (Muggleton *et al.*, 1996; Parker, 1998).

Levels of esterase resistance were above the long-term trends (2% S, 36% R_1 and 62% R_2 or R_3) in the 1,800 aphids analysed from England in 1997. However, most samples were collected during late summer and early autumn from vegetable brassicas with aphid control problems, and where insecticide use patterns would have provided a strong selection pressure for variants with elevated esterase. During the winter the proportion of R_2 and R_3 types in the population tends to decline due to their reduced ability to overwinter (Foster *et al.*, 1996).

MACE aphids were found at 14 of the 72 sites sampled (including 11 potato crops and 10 protected crops), always in combination with esterase resistance. Although some of these were from the MACE 'hot-spots' in Lincolnshire where major control problems had been encountered in 1996 (Foster *et al.*, 1998), MACE aphids were also found as far afield as Kent, Worcestershire and Lancashire, indicating a wider geographic spread. The overall proportion of MACE aphids was 26%, but since sampling had concentrated on sites with control problems, this is likely to be an overestimate of the true proportion of MACE in the 'national' population. MACE was not found in five samples from potatoes in eastern Scotland, although half of the 189 aphids analysed were R_2 or R_3, much higher than found previously.

Germany

Intensive and systematic sampling from 58 German potato crops showed that levels of esterase-based resistance (16% S, 38% R_1, 39% R_2, 7% R_3) mirrored closely those determined in a survey conducted in 1992 (Wege, 1994), indicating a trend for stability of this mechanism, as in the UK.

MACE was indicated in only 10 aphids from a total of 1,275 analysed but these were unusually associated with S levels of esterase, and the species was considered to have been misidentified. Indeed, *Aphis frangulae* and *A. nasturtii* were present in significant numbers in these crops and pirimicarb-insensitive acetylcholinesterase has been confirmed in these species

(unpublished); in fact, none has been found to date with sensitive acetylcholinesterase. Further sampling in 1998 will aim to establish categorically whether the MACE mechanism is present in Germany.

Greece

MACE was first found in Greece in 1991 in aphids sampled from peach near Meliki (Moores *et al*, 1994a). Control problems with pirimicarb had been apparent since 1988, following 10 years of intensive pirimicarb use both in peach and tobacco, the principal secondary host in this region for *M. persicae* and its tobacco-feeding form, *M. nicotianae* (Blackman, 1987).

Samples taken from Meliki in 1997 indicated MACE in 90% of aphids, distributed evenly amongst R_1, R_2 and R_3 types. Esterase levels remained high (5% R_1, 39% R_2 and 56% R_3) although apparently marginally lower than in 1990, when 90% were R_3 (Wege, 1994). These data indicate that MACE is inherently stable under holocyclic conditions in the absence of selection. Almost no pirimicarb has been used in the Meliki region since 1991.

Netherlands

MACE was first confirmed in Dutch glasshouses in 1993 following the intensive use of pirimicarb in IPM programmes over many years. In 1997, esterase levels were relatively low (8% S, 80% R_1, 6% R_2, 6% R_3) in the 64 aphids analysed from three glasshouses, but almost all resistant insects also had MACE. Although pirimicarb is still widely used in Dutch protected crops, as soon as resistance problems arise, growers switch to chloronicotinyl chemistry which is unaffected by either resistance mechanism, and can be applied in an IPM sensitive manner. This might be reflected in the relatively low esterase levels for glasshouse populations. Interestingly, MACE was absent in a sample of 84 aphids (2% S, 33% R_1, 65% R_2) from a glasshouse pepper crop, and in 21 aphids from a field crop of sugar beet in neighbouring Belgium.

Italy

In 623 aphids sampled from peach growing areas in central, northern and south-eastern Italy, esterase levels were high (2% S, 6% R_1, 23% R_2, 69% R_3), in line with previous years (Wege, 1994) and reflecting the difficulty in controlling *M. persicae* with OP and carbamate insecticides in these regions. Overall, MACE was present in about 40% of R_2 and R_3 aphids, although there was considerable variation (from none to 98%) between samples irrespective of region.

Other areas

The small number of aphids (142 from seven potato crops) collected from France appear to have been mis-identified as *M. persicae*, as discussed for some of the German samples. No MACE was found in samples from a USA tobacco crop (36 aphids, half R_1 and half R_2) and a Canadian potato crop (84 aphids, mainly R_2 or R_3).

Implications for resistance management

Data from these surveys suggest that MACE is well-established in southern Europe, and is becoming more widespread in northern European countries, including the UK. Its frequent coincidence with high levels of E4 or FE4 carboxylesterase resistance and *kdr* has profound implications for controlling *M. persicae*, since these mechanisms collectively render the majority of available aphicides ineffective. Against this background, the most intuitive and workable option in the short-term is to identify and exploit any alternative insecticides that remain effective against multi-resistant populations. Over most of Europe at present, the most obvious candidates are the chloronicotinyls, exemplified by imidacloprid, which is being approved for use on several *M. persicae* host plants as a foliar and/or systemic application.

However, the perception of imidacloprid as a panacea for existing resistance problems introduces a new and substantial resistance risk. Strains of *Myzus* sp. with tolerance to nicotine, probably as a result of feeding on tobacco, have already been shown to exhibit tolerance to imidacloprid (Devine *et al.*, 1996; Nauen & Elbert, 1997). More potent resistance to imidacloprid was reported in strains of the tobacco whitefly, *Bemisia tabaci*, from vegetable-growing regions of southern Spain, where this compound was also adopted as a 'cure-all' for widespread resistance to other control agents (Cahill *et al.*, 1996). It is clear therefore that longer-term resistance management recommendations for *M. persicae* cannot rely exclusively on the continued availability of novel and unresisted products. They should instead aim to retain chemical diversity by exploiting both conventional and new insecticides in the most rational and sustainable manner possible.

For highly adaptable pests such as *M. persicae*, successful management of resistance depends not only on stipulating the way that insecticides are deployed, but also on reducing the total number of treatments applied (Denholm *et al.*, 1998). The first and unquestionably most effective line of attack is therefore to minimise use of chemicals to the point at which recommendations for reducing or diversifying selection pressure become a viable proposition. Unfortunately, non-chemical options for controlling this pest are still very limited. Biological control of aphids has proved successful in glasshouse vegetable crops, and its extension to other protected cropping systems should be a matter of priority. Principles of crop hygiene aimed at reducing aphid infestations and disrupting breeding cycles apply to protected and field crops alike. Removal of weed hosts in and around glasshouses, isolation of seed crops of potatoes from potential virus and aphid sources (e.g. potato dumps and untreated ware crops), and early burning-off dates for potatoes all show considerable scope for preventing continuous breeding and minimising the survival of potentially resistant aphids.

With the intensification of resistance in *M. persicae*, accurate identification of aphid species has become a critical component of effective pest management. Species composition is likely to exert an increasingly important influence on product choice, and consequently on whether satisfactory control is achieved in the immediate and longer term. Although the choice of insecticides for use against *M. persicae* is becoming very limited, this is not necessarily the case with other, usually more easily controlled species that also infest *Myzus* hosts including *Macrosiphum euphorbiae* on lettuces and potatoes, and *Brevicoryne brassicae* on brassicas.

In cases when *M. persicae* is the primary target, the challenge is now to formulate insecticide use guidelines that not only maximise the likelihood of control in the short-term, but which also retard the accumulation of resistance mechanisms. This would clearly benefit from a knowledge of the resistance status of aphids at the time of spraying (Parker, 1998). In the absence of MACE, for example, pirimicarb not only remains one of the most effective compounds against other known mechanisms, but through its selective properties can contribute to preserving natural enemies and therefore reducing the need for successive treatments. If MACE is present, however, use of pirimicarb (or triazamate) will serve merely to select for MACE individuals and potentially promote their spread to other areas. Although the known resistance mechanisms in *M. persicae* can now be diagnosed precisely and relatively rapidly (e.g. Field *et al.*, 1997), current monitoring techniques require well-equipped laboratories and cannot realistically provide instant decision-making support for individual growers. However, there is unquestionably scope for developing simpler assays purely for disclosing the presence or absence of resistance to particular products (P. Wege, unpublished). In mainland UK, body colour can be used as a crude indicator of MACE, since the great majority of MACE aphids identified so far have been red in appearance. However, there is no constant association between colour and MACE, since aphids lacking this mechanism comprise both red and green morphs. Furthermore, this association does not apply in southern Europe where body colour and the presence of MACE occur as independent traits, presumably due to genetic recombination during sexual reproduction.

Work to evaluate and refine product use recommendations following the appearance of MACE is currently in progress at IACR-Rothamsted and elsewhere. In the UK, provisional resistance management guidelines for *M. persicae* will shortly be disseminated through the newly-formed Insecticide Resistance Action Group (IRAG), whose membership includes public sector researchers, consultants and agrochemical manufacturers. It is envisaged that these will need regular updating in the light of improved knowledge of the incidence, dynamics and practical implications of MACE, and its interaction with longer-established resistance mechanisms.

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REFERENCES

Blackman R.L (1987). Morphological discrimination of a tobacco-feeding form from *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), and a key to New World *Myzus* (*Nectarosiphon*) species. *Bulletin of Entomological Research* **77**, 713-730.

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

Denholm I; Cahill M; Dennehy T J; Horowitz A R (1998). Challenges with managing insecticide resistance in agricultural pests, exemplified by the whitefly, *Bemisia tabaci*. *Philosophical Transactions of the Royal Society, Series B* (in press).

Devine G J; Harling Z K; Scarr A W; Devonshire A L (1996). Lethal and sublethal effects of imidacloprid on nicotine-tolerant *Myzus nicotianae* and *M. persicae*. *Pesticide Science* **48**, 57-62.

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

Devonshire A L; Moores G D; Ffrench-Constant R H (1986). Detection of insecticide resistance by immunological estimation of carboxylesterase activity in *Myzus persicae* (Sulzer) and cross reaction of the antiserum with *Phorodon humuli* (Schrank) (Hemiptera: Aphididae). *Bulletin of Entomological Research*, **76**, 97-107.

Field L M; Anderson A P; Denholm I; Foster S P; Harling Z K; Javad N; Martinez-Torres D; Mores 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; Harrington R; Devonshire A L; Denholm I; Devine J; Kenward M G; Bale J S (1996). Comparative survival of insecticide susceptible and resistant peach potato aphids in low temperature field trials. *Bulletin of Entomological Research*. **86**, 17-27.

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

Martinez-Torres D; Devonshire A L; Williamson M S (1997). Molecular studies of knockdown resistance to pyrethroids; cloning of domain II sodium channel gene sequences from insects. *Pesticide Science* **51**, 265-270.

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

Moores G D; Devine G J; Devonshire A L (1994b). Insecticide resistance due to insensitive acetylcholinesterase in *Myzus persicae* and *Myzus nicotianae*. *Proceedings of the Brighton Crop Protection Conference - Pests and Diseases* **1**, 413-418.

Muggleton J; Hockland S; Thind B B; Lane A; Devonshire A L (1996). Long-term stability in the frequency of insecticide resistance in the peach-potato aphid, *Myzus persicae*, in England. *Proceedings of the Brighton Crop Protection Conference* **2**, 739-744.

Nauen R & Elbert A (1997). Apparent tolerance of a field-collected strain of *Myzus nicotianae* to imidacloprid due to strong antifeeding responses. *Pesticide Science* **49**, 252-258.

Parker W (1998). Keeping on top of resistance to aphicides. *Potato Review* **March 1998**, 12-15.

Wege P J (1994) Challenges in producing resistance management guidelines for *Myzus persicae*. *Proceedings of the Brighton Crop Protection Conference - Pests and Diseases*, 419-426.

The contribution of resistance in UK stored product pests to control failures and subsequent food contamination

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ABSTRACT

Levels of resistance to organophosphorus pesticides in stored products pests are now so high that some treatments are failing to control beetle pests and even accurate treatments at the full approved dose for admixture do not control mite strains found in grain stores. Mites are highly allergenic and have been found in 21% of cereal based foodstuffs purchased at retail outlets in the UK. The impact of contamination of food by both mites and the pesticides used to control them is discussed, together with ways of reducing contamination risks.

INTRODUCTION

Pesticides resistance has been recognised for many years as a factor in causing pests to survive control treatments (Georghiou & Saito, 1983). The development of resistance is generally time-linked and in its early stages, control can still be achieved under practical conditions. However, continued selection pressure will generally raise frequencies and levels of resistance to a point where control difficulties are experienced. While resistance in stored products pests (beetles, moths and mites) has been reported (Champ & Dyte, 1976) and the frequency and levels of resistance have been shown to increase with time (Champ, 1986; Muggleton, 1987), there is less information on control failures resulting from resistance. Clearly, where control techniques become less effective as a result of resistance, storage pest populations may increase and consequently cause greater contamination of foodstuffs.

Organophosphorus (OP) pesticides have dominated UK stored products pest control for some 30 years and pirimiphos-methyl is the market leader comprising over 75% of the total weight of pesticide used in UK grain stores in 1994/1995 (Norris & Garthwaite, 1997), with more than 12 tonnes of active ingredient used each year in grain store treatments and application direct onto stored cereal. For the last 20 years, only OP pesticides have been approved in the UK for application to stored grain and from 1987 to 1995, the Central Science Laboratory has collated data on the incidence of storage pests in a range of UK storage situations together with data on the resistance of such field strains.

Storage mites are strongly allergenic (Cuthbert *et al.*, 1979; Van Hage-Hamsten *et al.*, 1985; Stengard Hansen *et al*, 1996) and in recent years, seven cases of anaphylaxis and anaphylactoid reactions have been reported due to ingestion of mites from mite-contaminated food (Spiegel *et al.*, 1995; Castillo *et al.*, 1995; Scala, 1995; Matsumoto *et al.*, 1996 and Blanco *et al.*, 1997). Storage mites occur commonly throughout the UK grain industry (Prickett, 1992), infesting grain in store and they are frequently found in food manufacturing premises, warehouses and food stores including retail premises (B Thind - personal

communication). However, there has been little study of the presence of storage mites in UK foodstuffs and the possible risk to health that this might present.

METHODS AND MATERIALS

From 1987 to 1995, four information-gathering exercises were carried out to assess the incidence of storage pests in a range of storage situations, in farm grain stores (1987), commercial grain stores (1988/89), animal feed mills (1992) and oilseed stores (1995). Beetle and mite pests were collected from over 1000 premises and tested for resistance to pirimiphos-methyl. Discriminating doses were used to identify resistant strains (for example, saw-toothed grain beetles were contained for 5 hours on filter papers treated with a dose of 156 mg/m^2 of pirimiphos-methyl and flour mite strains were introduced to wheat treated with 8 mg/kg of pirimiphos-methyl and assessed for mortality after 14 days). Some strains of saw-toothed grain beetle, *Oryzaephilus surinamensis*, were further examined by exposing them to a range of doses of pirimiphos-methyl on wheat, at both 25°C and 10°C, to determine the dose of pesticide likely to be required to disinfest stored cereal.

In 1996, 567 samples of a range of cereal based foodstuffs (in eight general categories of baby food, biscuits, bread, breakfast cereal, cakes, flour, and food mixes) were purchased at retail outlets throughout England and Wales and analysed for the presence of storage mites using a modified flotation separation technique as originally described by Thind & Wallace (1984). Samples of 20 g of each foodstuff were analysed and any mites found were counted and identified, where possible, to species.

RESULTS

Primary beetle pests (from the pest genera, *Oryzaephilus, Cryptolestes and Sitophilus*) and storage mite pests (from the genera *Acarus, Glycyphagus, Lepidoglyphus and Tyrophagus*) were detected in all types of stores surveyed (see Table 1) and the most common beetle pest was the saw-toothed grain beetle which was found in 5% of farm grain stores, 20% of commercial grain stores and 46% of animal feed mills.

Table 1. Percentage of stores in which beetle and mite pests were found.

		Primary beetles *	Mites
1987	Farm Stores (742)†	10	72
1988/89	Commercial Stores (279)	27	81
1992	Animal Feed Stores (178)	68	89
1995	Oilseed Stores (109)	-	89

* *Oryzaephilus*, *Cryptolestes* and *Sitophilus* species
† (Number of stores surveyed)

The most frequently encountered storage mite species was the flour mite (*Acarus siro)*, which was found in 36% of farm stores, 59% of commercial grain stores, 63% of animal feed mills and 67% of oilseed stores. Testing of the collected strains of saw-toothed grain beetle and strains of flour mite showed large numbers of these pests to be resistant to the main storage pesticide in use in the UK, pirimiphos-methyl (see Table 2).

Table 2. Percentage of strains of saw-toothed grain beetle and flour mite resistant to pirimiphos-methyl in a range of storage situations.

		Oryzaephilus surinamensis	*Acarus siro*
1987	Farm Stores (742)	27	15
1988/89	Commercial Stores (279)	82	71
1992	Animal Feed Stores (178)	34	91
1995	Oilseed Stores (109)	-	93

While the dose used to discriminate resistant saw-toothed beetle strains from susceptible strains was only some 32% of the dose likely to be used in field applications, the discriminating dose used for flour mites was twice the maximum approved application rate for stored cereals in the UK.

The responses of the three main storage mite pests (*Acarus siro*, *Lepidoglyphus destructor* and *Tyrophagus putrescentiae*) to pirimiphos-methyl emuslifiable concentrate applied at 8 ppm to wheat, is shown in Table 3.

Table 3. Percentage of strains of storage mite species surviving twice the maximum approved dose of pirimiphos-methyl applied to wheat (14 day exposure).

	Year of sample	*Acarus siro*	*Lepidoglyphus destructor*	*Tyrophagus putrescentiae*
Farm stores	1987	14	0	0
Commercial stores	1989	71	11	100
Animal feed stores	1992	91	0	86
Oilseed stores	1995	93	17	97

While very few populations of *L. destructor* were designated as resistant by surviving 14 days exposure to twice the maximum permitted does of pirimiphos-methyl, the majority of strains of both *A. siro* and *T. putrescentiae* survived this test.

The small number of strains of saw-toothed grain beetle (collected from commercial stores and animal feed mills) which were exposed to ranges of doses of pirimiphos-methyl on wheat to determine likely doses (calculated as the dose to knockdown, KD, 99.9% of the test population measured in mg/kg) required to kill them in field admixtures on cereals, were all controlled at 25°C by the maximum field application rate of pirimiphos-methyl, 4 mg/kg. However, at 10°C, two out of the nine strains required doses higher then the maximum approved dose to kill them - one was controlled at 6.1 ppm and the other at 4.8 ppm (see Figure 1).

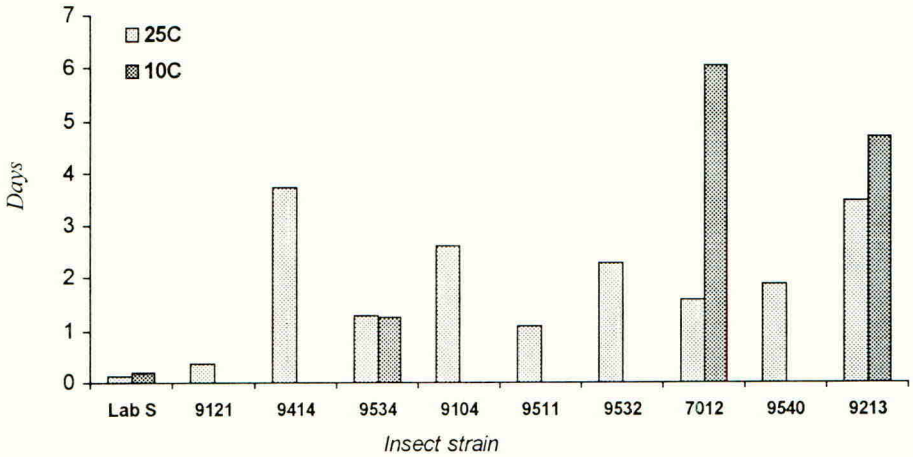

Figure 1. *Oryzaephilus surinamensis* exposed for 7 days at 10°C and 25°C to wheat treated with pirimiphos-methyl.

In the cereal-based food samples purchased at retail outlets, mites were present in all categories of food, with an average of more than one sample in five contaminated with mites (see Table 4). A wide range of mite species and genera were present and these included the main expected storage mite pest species but also some species of predatory mites (see Table 5).

Table 4. Contamination of freshly purchased cereal products by mites.

Product	Total Number of samples tested	% with Mites
Baby Food	61	15
Biscuits	71	20
Bread	113	25
Breakfast Cereal	118	19
Cakes	34	15
Flour	108	28
Food mixes	60	18
Other	2	50
Total	567	21

Table 5. List of mite species detected in cereal-based foodstuffs

Acarus farris	*Glycyphagus domesticus*
Acarus gracilis	*Glycyphagus* spp.
Acarus immobilis	Mite eggs
Acarus siro	*Pygmephoridae*
Acarus spp.	*Rhizoglyphidae* spp.
Astigmatic mite	*Steneotarsonemus* spp.
Cheyletus spp.	*Tarsonemidae* spp.
Dendroptus spp.	*Thyreophagus entomophagus*
Dermanyssus gallinae	*Tyrophagus longior*
Dermatophagoides pteronyssinus	*Tyrophagus palmarum*
Gamasid mite	*Tyrophagus putrescentiae*
Lepidoglyphus destructor	*Tyrophagus* spp.

DISCUSSION

Field populations of the predominant storage beetle pest, the saw-toothed grain beetle, are still likely to be controlled by effective residual applications of pesticide to stored grain, if the grain is stored relatively warm. However, in the cooler conditions commonly found in UK grain, disinfestation may not be possible even using the maximum approved dose of the market-leading grain protectant.

The incidence of resistance in saw-toothed grain beetles was greatest in commercial stores where pesticide application is routinely made to the grain and thus likely to present the

highest selection pressure to the pests present. The incidence of resistance in farm stores was lower (only some 9% of stored grain on farms is routinely treated with residual insecticides) but the 'intermediate' frequency of resistant strains found in saw-toothed grain beetles from animal feed stores might represent the greater likelihood of resistant strains being imported with raw materials, from countries where pesticide resistance is recognised as being a major problem.

It is likely that with selection for resistance continuing since the survey data was collected as well as further selection over the next few years, control failures will become more frequent and the excellent reputation of UK grain for quality will be threatened.

No previous information on the incidence of mites as food contaminants in the UK has been gathered. While many of the foodstuffs analysed were multi-ingredient, thus presenting a range of possible routes for mite contamination via the raw materials used in their manufacture, mites were found in 28% of flour samples, indicating that stored wheat and/or the subsequent milling process might be important sources of mite contamination. However, it must be stressed that there are a number of likely contamination routes, including the penetration of 'clean' manufactured goods in warehouses/stores or even in retail display units by residual populations of storage mites in the structures of such premises. More study is required to confirm the highest risk areas for mite contamination of cereal based foodstuffs.

The mite levels were generally low, with the detection level set at one mite per 20 g sample of foodstuff (equivalent, for example, to 75 mites present in a standard 1.5 kg retailed bag of flour) but several samples contained more than one mite. One sample of a dry cereal based baby food contained nearly 400 mites per 20 g sample. While this level of contamination may well be considered as totally unacceptable, it still falls some way short of the several hundred mites per gram reported by other workers as causing serious health problems associated with anaphylactoid reaction following ingestion of heavily contaminated food materials. Sanchez-Borges *et al.*, (1997) reported up to 5,000 mites per gram of flour. At the same time, while there is currently no legally defined level for 'acceptable' numbers of mites in foodstuffs, all food retailed in the UK must be fit for the purpose and legal decision would have to be made to decide whether it was, or was not, reasonable to expect a consumer to accept a food product contaminated with a certain level of storage mite presence.

The resistance of storage mites to the market-leading stored grain protectant (mites are also cross-resistant to all other currently approved grain protectants - A J Prickett, personal communication) is at such a level that two of the main species regularly found in grain stores can be considered 'proof' against the pesticides applied for their control. This may well result in a higher risk of contamination of foodstuffs by mites, as further evidence emerges of the total breakdown of chemical control options.

However, mites are not able to survive in commodities with an equilibrium relative humidity (erh) of less than 70% (which equates to some 14.5% moisture content in grain and 7.5% moisture content in oilseed rape). A greater use of cooling and drying equipment in the grain industry must be made if mite control is to be improved. There would seem to be little likelihood of a new acaricidal grain protectant being available in the UK for some years.

OP pesticides used to protect stored grain from infestation have been confirmed as surviving the storage period and manufacturing processes (Wilkin and Fishwick, 1981) and have been detected in a range of cereal based foodstuffs purchased at retail outlets (Anon., 1994; Anon., 1995). In 1993, 44% of samples of wholemeal bread contained residues of pirimiphos-methyl and while the 1996 equivalent data showed residues in only 23% of samples, it is considered likely that the use of residual pesticides has increased in subsequent years primarily as a result of difficult harvesting and storage conditions, leading to higher infestation risks. MAFF surveys have even shown that in 1993 and 1994, 10% and 18% respectively of organic bread samples were contaminated with OP residues; and in 1995 16% of infant cereals (rusks) were similarly contaminated. While the contamination of foodstuffs by an OP pesticide might be acceptable on the basis that storage mites were being controlled and thus themselves not contaminating the foodstuffs, the fact that many mite populations will simply not be controlled by residual admixture treatments suggests that the UK must radically re-think its storage strategies in the light of such high frequencies and levels of pesticide resistance.

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REFERENCES

Anon (1994). Ministry of Agriculture, Fisheries and Food and the Health and Safety Executive Annual Report of the Working Party on Pesticide Residues: 1993. Supplement to The Pesticides Register 1994, HMSO.

Anon (1995). Ministry of Agriculture, Fisheries and Food and the Health and Safety Executive Annual Report of the Working Party on Pesticide Residues: 1994. Supplement to The Pesticides Register 1995, MAFF Publications.

Blanco C; Quiralte J; Castillo R; Delgado J (1997). Anaphylaxis after ingestion of wheat flour contaminated with mites. *Journal of Allergy and Clinical Immunology* **99**, (3), 308-313.

Castillo S; Sanchez-Borges M; Capriles A; Suarez-Chacon R (1995). Systemic anaphylaxis after ingestion of mite-contaminated flour. *Journal of Allergy and Clinical Immunology* **95**, 304.

Champ B R (1986). Occurrence of resistance to pesticides in grain storage pests. In: *Pesticides and Humid Tropical Grain Storage Systems*, B R Champ and E Highley (Eds), ACIAR: Canberra.

Champ B R; Dyte C E (1976). Report of the FAO Global Survey of Pesticide Susceptibility of Stored Grain Pests, Rome: FAO.

Cuthbert O D; Brostoff J; Wraith D G; Brighton W G (1979). "Barn Allergy": Asthma and rhinitis due to storage mites. *Clinical Immunology* **9**, 229-236.

Georghiou G P; Saito T (1978). (Eds). Pest resistance to pesticides. Plenum, New York, 1983.

Muggleton J (1987). Insecticide resistance in stored product beetles and its consequences for their control. In: *Stored Products Pest Control*, T J Lawson (Ed), BCPC Monograph No. 37, Thornton Heath: BCPC Publications: 177-186.

Matsumoto T; Hisano T; Hamaguchi M; Miike T (1996). Systemic anaphylaxis after eating storage-mite-contaminated food. *International Archives of Allergy and Immunology* **109**, 197-200.

Norris L N; Garthwaite D G (1997). Pesticide Usage Survey Report 137: Farm Grain Stores in Great Britain 1994/5. London: MAFF. PB2946.

Prickett A J (1992). Recent surveys of post-harvest pest problems in farm and commercial grain stores in the UK. *Proceedings Brighton Crop Protection Conference- Pests and Diseases 1992*, **1**, 271-280.

Sanchez-Borges M; Capriles-Hulett A; Fernandez-Caldas E; Suarez-Chacon R; Caballero F; Castillo S; Sotillo E (1997). Mite-contaminated foods as a cause of anaphylaxis. *Journal of Allergy and Clinical Immunology* **99**, (6), 738-743.

Scala G (1995). House dust mite ingestion can induce allergic intestinal syndrome. *Allergy* **50**, 517-519.

Spiegel W A; Anolik R; Jakabovics E; Arlian L G (1995). Anaphylaxis associated with dust mite ingestion. *Annals of Allergy Asthma and Immunology* **74**, 56.

Stengard Hansen L; Herling C; Danielson C (1996). Densities of *Lepidoglyphus destructor* and levels of its major allergen Lep D I in grain and flour. *Proceedings of XX th International Congress of Entomology*, 569.

Thind B B; Wallace D J (1984). Modified flotation technique for quantitative determination of mite populations in feedstuffs. *Journal of the Association of Official Analytical Chemists* **67**, (5), 866-868.

Van Hage-Hansten M; Johansson S G O; Hoglund S; Tull P; Wiren A; Zetterstrom O (1985). Storage mite allergy is common in a farming population. *Clinical Allergy* and Immunology **15**, 555-564.

Wilkin D R; Fishwick F B (1981). Residues of organophosphorus pesticides in wholemeal flour and bread produced from treated wheat. *Proceedings British Crop Protection Conference - Pests and Diseases 1981*, **1**, 183-187.

Intracellular proteases: their role in insecticide toxicity and resistance mechanisms

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ABSTRACT

Protein metabolism constitutes a major physiological resource that can act as a compensatory mechanism under pesticidal stress. The degradation products of intracellular protein can be re-utilized in protecting the cell from stress. Recently, high intracellular protease activities have been found in a malathion-resistant strain of adults of *Musca domestica* L. These proteases are associated with tissues other than the gut and are responsible for metabolic processes of fundamental importance other than dietary protein digestion. In this review article, the advantages an insecticide resistant strain can have from this adaptive aspect of protein metabolism are suggested.

INTRODUCTION

Of all enzymes, proteases are the most widely distributed, in all living cells. Proteases, which hydrolyse the peptide bond, are involved in protein digestion outside of cells and also in the expression and regulation of cell processes. In a strict sense, these intracellular proteases degrade exclusively resident cellular proteins and peptides although proteins that are transported into the cell are also degraded. Intracellular proteases are either exopeptidases which act at the amino- or carboxyl terminal or endopeptidases which are capable of cleaving peptide bonds in the central region of polypeptides. Exopeptidases acting on amino- or carboxyl-termini of the amino acids are known as aminopeptidases or carboxypeptidases, respectively. The exopeptidases act on short chain peptides that are produced by the action of endopeptidases, thus acting as polymer terminal enzymes as part of a cascade effect in protein catabolism. Proteases can achieve very rapid changes in the levels of short-lived proteins such as regulatory enzymes. Protein turnover (metabolism) also provides a means of cell or/ tissue adaptation to the changes in metabolic conditions by changing the protein level. It enables the cells to adapt to various needs imposed by diet, hormone and environmental factors. The multitude of processes in which cellular proteases participate has made it difficult to identify the functions of these proteases adequately. Nevertheless, cellular proteases function to (a) create biologically active molecules, or (b) destroy biologically active proteins and peptides (Bond & Butler, 1987; Rivett, 1990).

Insects, like other living organisms, require proteases for degrading proteins and peptides. In insects, proteases are involved in specialized functions related to their particular physiology

as well as in digestion of dietary proteins, in embryogenesis and reproduction, and in growth and development (Law *et al.*, 1977). Proteases, endo-and exopeptidases in an insect's digestive system belong to one of the groups based on catalytic residues at their active sites; hydroxyl (serine), thiol (cysteine) and carboxyl (aspartic) proteases. The best known digestive serine proteinases, trypsin and chymotrypsin, have been identified in numerous insects as major components of digestive fluids. Proteolytic enzymes, mostly lysosomal (acid) hydrolases present in the yolk sphere, have been described as responsible for yolk protein degradation during embryogenesis in insects (Terra & Ferreira, 1994; Izumi *et al.*, 1994). Besides proteases involved in digestion, reproduction, and development, intracellular proteases (cytoplasmic and lysosomal) responsible for general protein turnover also form an important feature of an insect life. However, the study of cellular proteases in insect physiology is very fragmentary, but recent reports have highlighted their relationship with insecticide resistance in insects and this paper reviews these discoveries.

PROTEASES AND INSECTICIDE TOXICITY

Knowledge of the effects of insecticides on cellular proteases is limited. The insecticidal activity of the crystal endotoxin of *Bacillus thuringiensis* is due to its binding to brush border membrane aminopeptidase-N in the midgut of *Lymantria dispar*, and *Plutella xylostella*.

Proteolytic enzymes hold little promise as a target for conventional insecticides. Proteases cannot degrade insecticide molecules because of their usual lack of an amide bond. Serine proteases have been reported to hydrolyze α-naphthyl acetate, a substrate used widely to measure non-specific esterases. However, the hydrolysis of ester-containing insecticides, phosphorothiolates, phosphates, carbamates and pyrethroids by these proteases does not occur. An intracellular protease (Dipeptidyl aminopeptidase II) has been shown to hydrolyze a dipeptide ester (McDonald & Schwabe, 1977). The esterolysis potential of proteinases (trypsin-like) in catalyzing the synthesis of peptides appears to be restricted to substrates of the type in which amino moeity is present. Most insecticides have no structures similar to these and there seems to be no involvement of proteases in the hydrolysis of insecticide esters.

Proteins, prominant among the molecules essential to life, are the key organic constituents of cells. The regulation of protein synthesis is correlated with protein degradation and both protein synthesis and degradation comprise protein turnover. Recognizing that there are differences in rates, all proteins turnover continuously. Protein degradation also responds to changes in the metabolic state of cells and tissues. Insecticides interact with protein as inhibitors of acetylcholinesterase or they can bind to proteins (e.g. sodium channels in the nervous system) and bring about a change in the normal functioning of these proteins. The insects respond to these actions (a) by induction of insecticide metabolizing enzymes (IME) and (b) by alterations in metabolic systems which can compensate the toxic action of insecticides. There is a role of intracellular proteases in these mechanisms but it is not well-characterized.

Depletion in protein contents and increase in free amino acids with relative increases in transaminases and enzymes of energy cycles after exposure to sublethal concentrations of various classes of insecticides has been observed. These alterations were considered as a compensatory mechanism during intoxication by insecticides (Phillip & Rajasaree, 1996). The results of insecticide interaction with protein metabolism at sublethal concentrations demonstrated the similar patterns in insects as were observed in the aquatic ecosystems (Shakoori *et al.*, 1993; Tufail & Shakoori, 1994).

PROTEASES AND INSECTICIDE RESISTANCE

Resistance to insecticides in many pest insect species is governed respectively by qualitative and quantitative changes in insecticide target enzymes and IME. Nearly all the classes of insecticides induce enzymes which can degrade them (Terriere, 1984). The induction is *de novo* synthesis of enzymes. The relationship of induction of IME with the intracellular proteolytic enzymes is not known. The adaptive nature of insecticide-resistant insect strains to tolerate the high doses implicates the potential of a strain to induce these enzymes. We, therefore, suggest that induction of IME is associated with modification of protein metabolism directed to enzyme synthesis. Resistance is a genetic change in response to insecticides, which select the individuals capable of tolerating high doses of an insecticide. As the selection proceeds a strain evolves equipped with sufficient IME levels to accelerate insecticide metabolism. The complementary enzyme levels associated with these changes are usually overlooked. One such example has been observed in *Tribolium castaneum* and *Musca domestica*, where respectively total proteases (azocasein hydrolyzing enzymes) and intracellular proteases (cytoplasmic and lysosomal enzymes) exhibited high activities in a malathion-resistant strain of each species compared with an insecticide susceptible strain (Saleem *et al.*, 1994). Subsequent studies on the interaction of insecticides with intracellular proteases revealed alterations of these activities following malathion exposure of resistant and suceptible strains. *In vivo* and *in vitro* analyses of these proteases following primiphos-methyl and tri-O-cresyl phosphate treatment showed inhibition of some of the enzymes in the rat liver and human brain tissues, respectively (Mantle *et al.*, 1997; Saleem *et al.*, 1998).

PROTEASE ACTIVITIES IN INSECTS; INSECTICIDE RESISTANT AND SUSCEPTIBLE STRAINS

Protease activities in many of the life stages of a DDT resistant, 17bb strain (R) (Figure 1) were high compared with the susceptible, Cooper strain (S). The larva exhibited higher activity than pupa and adults, which may be partly due to greater expression in the larval gut. The larvae lacked proline endopeptidase activity in both strains. A similar pattern of protease activities was observed in a fenitrothion-resistant strain (571ab) (data not shown). Activities of proteases in the body regions, i.e., head, thorax and abdomen in both sexes of 17bb and Cooper, being highest in the abdomen showed increased levels in 17bb compared with Cooper. The activities of some enzymes were below detection limits in the head and thorax of 17bb and Cooper strains whereas in the abdomen a complete set of enzymes was recorded as in the whole fly homogenate.

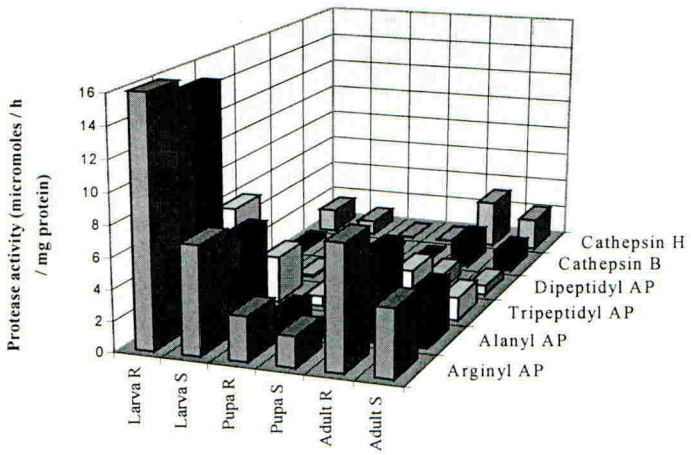

Figure 1. Protease activities (aminopeptidase, AAAP0 in life stages of resistant (R), 17bb and susceptible (S) Cooper strains.

RESPONSE TO INSECTICIDE TREATMENT IN RESISTANT AND SUSCEPTIBLE STRAINS

Following *in vivo* topical treatment with DDT of a DDT-resistant strain, alanyl aminopeptidase and arginyl aminopeptidase activities increased significantly and rapidly at 1, 2, 3 and and 24 h after treatment compared with control activities. After 24 h, the activity of most proteases returned to that of the control (flies treated with acetone only after 24 h; no mortality). The dead flies 24 h after treatment had lower activities than the live ones in all cases. In insects exposed to fenitrothion, the pattern of activity over the subsequent 24 h period varied depending on the individual protease. However, corresponding enzymes shared broadly similar pattern of activity changes in both the resistant and susceptible insect strains. The changes in activities of total cytoplasmic and lysosomal proteases after fenitrothion treatment in 571ab strain are shown in Figures 2 and 3.

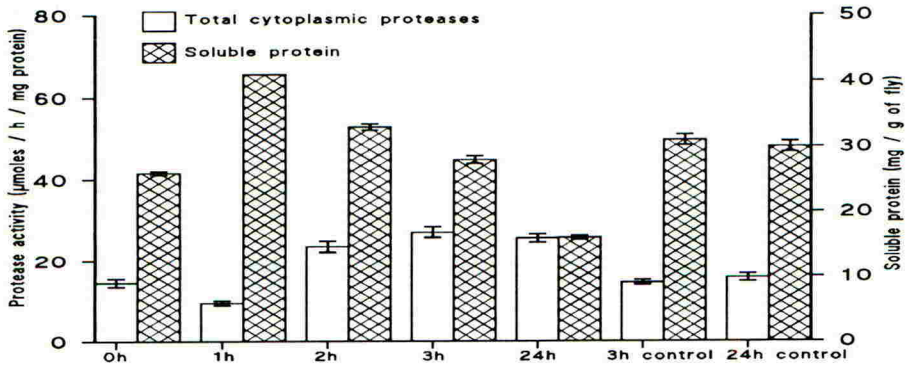

Figure 2. Total cytoplasmic protease activities (means ± SE) and soluble protein after fenitrothion treatment.

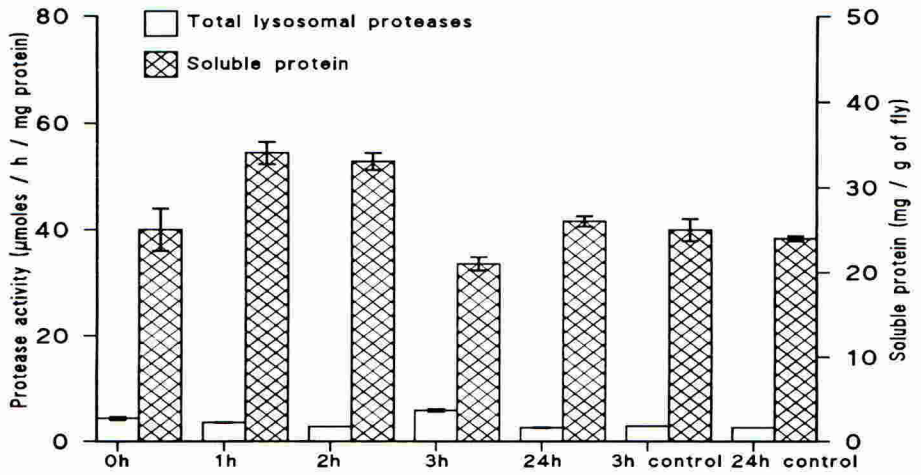

Figure 3. Total lysomal protease activities (means ± SE) and soluble protein after fenitrothion treatment.

DISCUSSION

The variability of intracellular proteases in all life stages of resistant and susceptible strains of *M. domestica* re-inforce the idea of biochemical variability among strains as a rule rather than the exception. The re-allocation of resources for metabolic and developmental processes during the selection by insecticides affect the biochemical functions of the resistant strain.

High intracellular protease activities in the resistant strains may enable them to adapt to the new environment (insecticide) during insecticide selection by having more than one compensatory mechanisms to overcome the effects of insecticides and also to help reduce the metabolic costs associated with this adaptation.

Both resistant and susceptible strains showed increased protease levels upon lethal insecticide treatment. There was a trend in the magnitude of the increase which was larger in the susceptible strain compared to the resistant, which already had a high level. This protease activity was inversely related with protein contents of both strains following treatment with fenitrothion but this did occur with DDT. Hence, it is the endogenous level of proteases which is important in the resistance mechanism, rather than the capacity of the strains to increase the activity under insecticidal stress. However, cytoplasmic proteases increased more than lysosomal proteases in insect treated with fenitrothion or DDT, suggesting a more important role for cytoplasmic proteases in protein turnover which provide the precursor amino acids for protein synthesis Following fenitrothion or DDT treatment, lysosomal proteases returned to baseline (or lower) values at 24 hours after a period of elevated activity, in surviving flies. Soluble protein content of the fly followed a similar pattern of increase and decrease but in advance of the proteases. Thus at 24 hours there is conservation of proteins rather than degradation.

We, therefore, suggest that the role of increased protease activities in resistance mechanism is to provide an increased supply of precursor amino acids, from proteolytic degradation, for the synthesis of detoxifying enzymes.

REFERENCES

Bond J S; Butler P E (1987). Intracellular proteases. *Annual Review of Biochemistry* **56**, 333-364.

Izumi S; Yano K; Yamamoto Y. and Takahashi S Y (1994). Yolk protein from insect eggs-structure, biosynthesis and programmed cell degradatiuon during embryogenesis. *Journal of Insect Physiology* **40** (9), 735-746.

Law J; Dunn P E; Kramer E J; (1977). Insect proteases and peptidases. *Advances in Enzymology* **45**, 389-425.

Mantle D; Saleem M A; Williams F M; Wilkins R M; Shakoori A R (1997). Effect of primiphos-methyl on proteolytic enzyme activities in rat heart, kidney, brain and live tissues *in vivo*. *Clinica Chem. Acta* 262, 89-97.

McDonald J K; Schwabe C (1977). Intracellualr exopeptidases, pp. 311-391. In, (Barret, A. J., ed), Proteinases in mammalian cells and tissues. North Holland, Amsterdam

Phillip G; Rajasaree B (1996). Action of cypermethrin on tissue transamination during nitrogen metabolism in *Cyprinus carpio*. *Ecotoxicology and Environmental Safety* 34 (2), 174-180.

Rivett A J (1990). Intracellular protein degradation. Essays in Biochemistry **25**, 39-81.

Saleem M A; Shakoori A R; Wilkins R M; Mantle D. 1994. In vivo effect of lambda-cyhalothrin on the proteolytic enzymes of malathion-resistant and susceptible strains of *M. domestica* L. Pakistan Journal of Zoology **26** (4), 327-333.

Saleem M A; Williams M F; Wilikins R M; Shakoori A R; Mantle D (1998). Effect of Tri-O-Cresyl Phosphate (TOCP) on proteolytic enzyme activities in mouse liver *in vivo*. *Journal of Environmental Pathology, Toxicolgy and Oncology* **17** (1), 69-73.

Shakoori, A. R., M. Z. Malik and M. A. Saleem, 1993. Toxicity of Karate to malathion resitant Pakistani strain of red flour beetle (*T. castaneum*) adults. *Pakistan Journal of Zoology* **25** (3), 261-271.

Terra W R; Ferreira, C (1994). Insect digestive enzymes: properties, compartmentalization and functions. *Comparative Biochemistry and Physiology* **109B** (1), 1-62pp.

Terriere L. C. (1984) Induction of detoxifying enzymes in insects. *Annual Review of Entomology* **29**, 71-78.

Tufail N; Shakoori A R (1992). Alphamethrin toxicity in *Tribolium castaneum* larvae. *Pakistan Journal of Zoology* **24** (1), 59-70.

Sensitivity of *Phytophthora infestans* to fluazinam and its use in potato blight control in Northern Ireland

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ABSTRACT

Fluazinam, a non-systemic protectant fungicide, has been used for the control of potato late blight in Northern Ireland since its introduction in 1994 and is currently applied to *c.* 20% of the crop area. A zoospore motility assay was used to test the sensitivity to fluazinam of isolates of *Phytophthora infestans* obtained from Northern Ireland potato crops between 1993 and 1997. All isolates proved very sensitive to fluazinam with zoospore motility completely inhibited by 20 - 60 μg fluazinam/litre. There was no consistent trend in sensitivity between isolates obtained from crops in different years, although fluazinam usage increased annually over the period examined. Fluazinam sensitivity was not affected by use of fluazinam or other fungicides on the crops from which isolates were obtained nor was there any association with phenylamide resistance or potato cultivar.

INTRODUCTION

Fluazinam has been approved in the UK since 1994 for the control of potato late blight. It has proved very effective in protecting foliage and, particularly, tubers from infection by *Phytophthora infestans* (Tucker *et al.*, 1994). Fluazinam is marketed in formulations which contain no other active ingredient and these are recommended for season long use on potatoes, with a maximum of 10 applications per crop. The possibility of the development of fungicide resistance is of particular concern in the case of potato blight. *P. infestans* is a rapidly reproducing air-borne pathogen, which has a demonstrable ability to respond to environmental selection pressures such as host-plant resistance or fungicide usage. Subjecting it to repeated applications of a single fungicide within one season has the potential to select for fungicide resistant strains if viable, fit strains can develop. Zeneca have developed a technique for testing the sensitivity of *P. infestans* isolates to fluazinam based on inhibition of zoospore motility. In view of the increasing use of fluazinam on potato crops in Northern Ireland, it was decided to use this to test the sensitivity of *P. infestans* isolates collected over the last five years.

MATERIALS AND METHODS

Source of *Phytophthora infestans* isolates

Samples of infected potato foliage together with data on sample site, potato cultivar, fungicide usage and disease incidence were obtained (mainly from seed crops) by members of the Department of Agriculture's Potato Inspection Service, as previously described (Cooke & Penney, 1992). Isolates were derived by bulking together the sporangia obtained from all

foliage samples within a single crop. At the end of each season, Inspectors provided estimates of fungicide usage for all seed potato crops in their areas.

Characterisation of isolates for phenylamide resistance and mating type

For 2-3 months after receipt, isolates were maintained on detached glasshouse-grown potato leaflets free from fungicide treatment. During this time, they were tested for phenylamide resistance using the floating leaf disc technique (Cooke, 1986) on 100 and 2 mg metalaxyl/litre. Isolates were designated resistant if they sporulated on 100 mg metalaxyl/l-treated discs. Isolates were subsequently transferred into pure culture and tested for mating type (Cooke *et al.*, 1995).

Sensitivity of isolates to fluazinam

The sensitivity assay was modified from an unpublished protocol developed by the University of Wageningen for Zeneca and now used by Zeneca as their standard protocol. Sporangial suspensions (*c.* 10^5 sporangia/ml) were prepared by flooding agar cultures of the appropriate *P. infestans* isolates with sterile distilled water. Suspensions were incubated at *c.* 5°C for 2-3 h to stimulate zoospore release.

Serial dilutions of fluazinam in distilled water were prepared from 'Shirlan' (Zeneca, 500 g fluazinam/litre) and 50 µl aliquots pipetted into the wells of 96-well ELISA plates. Subsequently, 50 µl aliquots of the appropriate sporangial suspension were added to each well to give final concentrations of 70, 60, 50, 40, 30 and 20 µg fluazinam/litre. Two replicate wells per isolate were used for each concentration and water controls were included. All solutions and ELISA plates were chilled to 5°C before use in order to maintain zoospore motility. Plates were incubated at 5°C for 1 h, then zoospore motility was assessed on a scale of 1-3, where 1 = very motile, 2 = motile, 3 = not motile. Results were expressed in terms of the minimum inhibitory concentration (MIC), which was defined as the lowest concentration which completely inhibited zoospore motility. Each isolate was tested at least twice.

To determine the optimum incubation time in order to achieve reproducible results and to maximise the number of isolates which could be tested, time-course experiments were carried out. Fluazinam concentrations from 10-100 µg/litre were tested against three selected isolates and zoospore motility assessed after 2, 10, 20 and 40 minutes and 1, 1.5, 2, 3, 4 and 5 h. The experiment was repeated twice.

RESULTS

Fluazinam usage on seed potato crops

Fluazinam was not used on commercial potato crops in Northern Ireland before 1994. Since then, usage has increased annually (Table 1). In 1997, just under 20% of seed crops received fluazinam applications for most of the spray programme. Additionally, other seed crops may have received one or two applications of fluazinam in mixed blight control programmes. This is borne out by data from the Survey of Pesticide Usage in Northern Ireland. In 1996, the most recent year surveyed, approx. 23% of seed crops, 9% of early ware and 17% of maincrop ware received one or more fluazinam applications (Jess *et al.* 1998).

Table 1. Fungicide usage on seed potato crops for most of the season.

Fungicide applied	Growers (%) using fungicides in				
	1993	1994	1995	1996	1997
mancozeb alone	70	69	63	61	60
fluazinam	0	5	13	16	19
phenylamide + mancozeb	17	14	14	14	12
other	13	12	10	9	9

Effect of incubation time on the sensitivity to fluazinam of the motility of *Phytophthora infestans* zoospores

In initial experiments, zoospore motility was assessed on a 1-4 scale, with three categories for different degrees of motility, in addition to not motile. However, it was found that this made assessments more time-consuming and less reproducible, so a 1-3 scale was substituted.

In a typical experiment (Table 2), sensitivity, as indicated by zoospore motility, increased with time of exposure. After 5 hours' incubation, zoospore motility in the water control treatments of some isolates had begun to decline, so a longer incubation was not considered appropriate. Sensitivity increased rapidly during the initial period of incubation (2 - 40 min), but changed relatively slowly between 40 min and 2 h incubation. An incubation time of one hour was therefore selected to give the most reproducible results. This also permitted up to 20 isolates to be tested at a time. The initial groups of isolates tested had MIC values of *c.* 40 µg/l, indicating that their zoospore motility was highly sensitivity to fluazinam.

Table 2. Effect of incubation time on sensitivity of zoospore motility of three isolates of *Phytophthora infestans* to fluazinam.

Incubation time (min)	MIC value (µg/l) Isolate 21/94	32/94	43/94	Isolate mean
2	80	80	70	77
10	80	60	50	63
20	60	50	50	53
40	50	40	40	43
60	40	40	40	40
90	40	40	40	40
120	35	40	40	38
180	35	20	30	28
240	25	20	25	23
300	20	10	15	15

Sensitivity to fluazinam of zoospore motility of isolates of *Phytophthora infestans* from Northern Ireland, 1993-1997

A collection of 90 isolates of *P. infestans* obtained from Northern Ireland crops between 1993 and 1997 was tested for sensitivity to fluazinam. Analysis of variance of the MIC values from the replicated sensitivity tests indicated that there was no significant difference in MIC value between isolates (P=0.529). Differences were accounted for by variation between tests, which may have resulted minor variations in fungicide concentration due to pipetting error and from differences in inoculum viability. The MIC values for individual isolates ranged from 20 to 60 µg fluazinam/litre. There was no evidence of any trend in sensitivity between isolates obtained from potato crops in different years (Table 3).

Table 3. Sensitivity to fluazinam of Northern Ireland *Phytophthora infestans* isolates collected in different years.

Year isolated	No. of isolates tested	MIC value (µg/l)	
		Range	Mean
1993	6	20 - 30	23
1994	27	20 - 50	28
1995	18	20 - 60	28
1996	24	20 - 50	38
1997	15	20 - 50	31
Total	90	20 - 60	31

The sensitivity of isolates obtained from crops which had received one or more fluazinam applications was compared with those from crops treated with other fungicides (Table 4) and found to be very similar. There was no association between phenylamide resistance and fluazinam sensitivity (Table 4) or between the potato cultivar from which isolates were obtained and sensitivity (Table 5).

Table 4. Sensitivity to fluazinam of Northern Ireland *Phytophthora infestans* isolates: the effect of fungicide usage and phenylamide resistance.

Character	No. of isolates tested	MIC value (µg/l)	
		Range	Mean
Fungicide usage			
No fluazinam	82	20 - 60	31
Fluazinam	8	20 - 40	26
Phenylamide resistance			
R	19	20 - 50	29
S	71	20 - 60	31

Table 5. Sensitivity to fluazinam of Northern Ireland *Phytophthora infestans* isolates: the effect of cultivar.

Cultivar	No. of isolates tested	MIC value (μg/l)	
		Range	Mean
Arran Banner	8	20 - 50	36
British Queen	7	20 - 50	35
Désirée	4	20 - 50	37
Dunbar Standard	5	20 - 50	33
Kerr's Pink	24	20 - 50	29
Nicola	4	20 - 50	34
Pentland Dell	4	20 - 30	24
Up-to-date	6	20 - 30	24
Other potato	27	20 - 60	30

In addition to the *P. infestans* isolates from potato, one isolate from tomato (cv. Gardener's Delight) was tested. Its sensitivity (MIC value 28 μg fluazinam/litre) was similar to that of the potato isolates.

Isolates of the A2 mating type of *P. infestans* are infrequent in Northern Ireland (Cooke *et al.*, 1995) and all but three were of the A1 mating type. The A2 isolates (obtained from two 1993 crops and one 1994 crops) had a mean MIC value of 23 μg fluazinam/litre (range 20-30 μg/l).

DISCUSSION

Prediction of the likelihood of the development of pathogen strains with stable resistance to novel fungicides is problematic. Fluazinam is currently the only example of the diarylamine group in widespread use, so it is not possible to extrapolate from the behaviour of related fungicides. Its application to potatoes to control late blight as a sole active ingredient with a recommendation for season-long use would be likely to select for fluazinam-resistant strains of *P. infestans* if viable strains can arise. However, mancozeb has been used in a similar way for the past 30 years with no recorded cases of resistance.

Practical fungicide resistance leading to disease control problems has generally occurred with single-site, systemic fungicides. Fluazinam is non-systemic and acts as an uncoupler of fungal oxidative phosphorylation (Guo *et al.*, 1991). It has been proposed that, as its action is non-specific, selection of resistant strains is extremely unlikely (Tucker *et al.*, 1994). Nonetheless, monitoring of the sensitivity of *P. infestans* populations exposed to fluazinam is desirable.

The zoospore motility test developed by Zeneca provides a rapid method for sensitivity testing. Since the zoospore phase of the pathogen is probably the most energy expensive, it is likely to be the most sensitive to a fungicide such as fluazinam which interferes with energy production. However, effects on zoospore motility should be reflected in the overall sensitivity of the pathogen during infection, since all steps of the infection process require energy production. The test is best-suited to use with *P. infestans* isolates in pure culture, but

could be applied to isolates maintained on potato tissue, provided that they were not excessively contaminated. In our initial tests, difficulties were experienced in obtaining consistent results. It was found crucial to ensure that that all suspensions were kept chilled to *c.* 5°C, otherwise zoospore motility rapidly declined.

In the Netherlands, where fluazinam has been used intensively since 1992, *P. infestans* isolates collected between 1993 and 1995 were tested using this technique and all proved very sensitive to fluazinam (Anon., 1997). In the sensitivity tests reported here, zoospore motility of all isolates tested was highly sensitive to fluazinam with MIC values most commonly between 30 and 40 µg/litre, in agreement with the figure of 40 µg/litre reported by Tucker *et al.* (1994). There was no evidence of any change in sensitivity over time or any association with any other factor. *In vivo* sensitivity to fluazinam was indicated by field trials inoculated with phenylamide-resistant and -sensitive isolates of *P. infestans* obtained from Northern Ireland crops in the preceding seasons. Fluazinam programmes achieved good control of foliage and tuber blight, better than mancozeb at the same interval (Cooke & Little, 1998). However, while this lends support to the view that fluazinam resistance may not be a problem in practice, it would be advisable to continue monitoring in view of the widespread use of this fungicide on potato crops and the potential for crop loss should late blight control fail.

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REFERENCES

Anon. (1997). In *Fungicide resistance in potato pathogens: potato blight* Phytophthora infestans. Slawson D D (ed), British Potato Council, FRAG-UK, pp 10.

Cooke L R (1986). Acylalanine resistance in *Phytophthora infestans* in Northern Ireland. *Proceedings of the 1986 British Crop Protection Conference - Pests and Diseases* **2**, 507-514.

Cooke L R: Little G (1998). Potato blight and its control. In *An integrated approach to crop production*, Easson D L (ed), Agricultural Research Institute of Northern Ireland, Occasional Publication No. 27, pp. 39-46.

Cooke L R; Penney R E (1992). Phenylamide resistance in *Phytophthora infestans* in Northern Ireland - a changing situation. *Proceedings Brighton Crop Protection Conference - Pests and Diseases - 1992*, **1**, 201-206.

Cooke L R; Swan R E; Currie T S (1995). Incidence of the A2 mating type of *Phytophthora infestans* on potato crops in Northern Ireland. *Potato Research*, **38**, 23-29.

Guo Z J; Miyoshi H; Komyoji T; Haga T; Fujita T (1991). Uncoupling activity of a newly discovered fungicide, fluazinam [3-chloro-N-(3-chloro-2,6-dinitro-4-trifluoromethyl-phenyl)-5-trifluoromethyl-2-pyridinamine]. *Biochimica et Biophysica Acta*, **1056**, 89-92.

Jess S; Kidd S L B; McCallion T (1998). Pesticide Usage Survey Report 146, Northern Ireland Arable Crops 1996. HMSO, Belfast, in press.

Tucker R P; Leaper D J; Laidler S T (1994). Fluazinam - control of potato late blight - UK experience 1992-3. *Proceedings Brighton Crop Protection Conference - Pests and Diseases - 1994*, **1**, 295-300.

Studies comparing the sensitivity of European and USA isolates of *Phytophthora infestans* to propamocarb hydrochloride

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ABSTRACT

An ongoing propamocarb sensitivity monitoring programme based on inhibition of sporangiophore production for European isolates of *Phytophthora infestans* has now been supplemented with isolates from the USA. European isolates collected in 1995 and 1996 showed no change in sensitivity to propamocarb and isolates collected in 1997 had increased sensitivity. USA isolates from states other than California had a higher sensitivity to propamocarb than European isolates. Californian isolates had a lower sensitivity, however in the more discriminatory spore viability test, Californian isolates were no different to a highly sensitive European isolate in their sensitivity to propamocarb. There was no evidence of resistance to propamocarb in any of the European or USA isolates.

INTRODUCTION

Propamocarb hydrochloride (propyl 3-(dimethylamino) propylcarbamate) is a systemic fungicide used in the control of oomycete pathogens in a variety of crops both in the horticultural and agricultural markets. In the early 1990's propamocarb in combination with either mancozeb ('Tattoo'®) or chlorothalonil ('Tattoo C'®) was introduced into the European market for the control of potato late blight (*Phytophthora infestans*). In 1995, propamocarb plus chlorothalonil was granted emergency use clearance in the USA following the widespread incidence of phenylamide-resistant strains.

Following the introduction of propamocarb for use in spray programmes to control potato late blight, a sensitivity monitoring programme was instigated at the University of Wales, Bangor using isolates collected in Europe (Bardsley *et al.* 1996). The results from 1994/1995 showed that there had been no shift in the sensitivity of *P. infestans* to propamocarb following its introduction. Additionally, there was no difference in the sensitivity of phenylamide sensitive and insensitive strains or between the A1 and A2 mating types. This programme is continuing and remains an important element in an anti-resistance strategy for propamocarb. In 1997, the programme was extended to include isolates from the USA, where propamocarb is widely used in late blight control.

P. infestans populations in Europe and the USA are known to differ markedly in their genetic make-up, and many of the USA races are not encountered in Europe. The populations in the USA are also more genetically diverse with new races appearing frequently, and are dominated by A2 mating type isolates, which have been reported to be faster growing and

more aggressive pathogens than the USA A1 mating types (Secor *et al.* 1995). In England and Wales, by contrast, isolates of A1 and A2 mating type have not been shown to differ in aggressiveness, and A2 isolates tend to occur at a much lower frequency than A1 isolates (Day & Shattock, 1997)

MATERIALS AND METHODS

European isolates collected from the field as infected leaves, stems or tubers were cultured initially on rye A agar supplemented with rifamycin, ampicillin and nystatin, which ensured that all bacterial contamination was removed. Isolates were then transferred to unamended rye A agar before being transferred to potato leaves. Potato plants (cv. Home Guard) had been grown in 5 litre pots with John Innes number 1 compost, at 18°C and using a photoperiod of 16h per day. USA isolates were supplied from three collaborating laboratories in the USA on rye agar.

A leaf disk assay was used to determine the sensitivity of the isolates (Sozzi *et al.*, 1992). Replicates of 5 leaf disks (13 mm diameter) of sensitive potato cultivar Home Guard were floated on solutions of propamocarb at the concentrations of 0, 10, 50, 200 and 500 ppm a.i., each in a separate well of a square 25-well repli-dish. Prior to infection of the leaf disks, each isolate was passaged through Home Guard leaves to ensure that the isolates remained pathogenic. These leaves were used to prepare a suspension of 2-3 x 10^4 sporangia/ml, which was then incubated for 2-3 hours at 10°C to induce release of zoospores. Leaf disks were then inoculated with 20µl of the suspensions and incubated at 18°C for 7d using a photoperiod of 16h per day.

Sporulation was scored using the following scale:

Score	Observation
0	sporangiophores absent
1	1-4 sporangiophores per disk
2	5-12 sporangiophores per disk
3	moderate sporulation; sporangiophores visible under binocular microscope
4	profuse sporulation visible with the naked eye

European isolates in 1996 were obtained from the United Kingdom (98 isolates) and the Republic of Ireland (21 isolates). In 1997, 109 European isolates were sourced entirely from the UK. It was not recorded which of these isolates had been exposed to propamocarb.

USA isolates tested in 1997 were originally isolated over several years:

Year	Number of isolates
1992	1
1994	26
1995	3
1996	28
1997	6

The 0-4 scoring system made median values more appropriate than mean values to summarise the raw data. Statistical analysis of the data was carried out using the Mann Whitney test (Mann & Whitney, 1947).

Spore viability tests were carried out by collecting spores from leaf disks treated at 0, 50 and 500 ppm and inoculating fresh untreated disks with the harvested spores. The fresh disks were incubated for one week and then assessed using the scoring system described above.

RESULTS AND DISCUSSION

European Isolates

The data for European isolates are summarised in Figure 1.

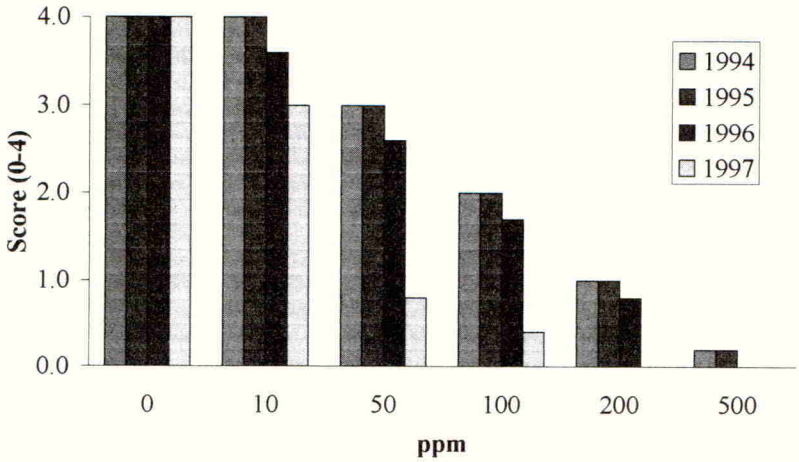
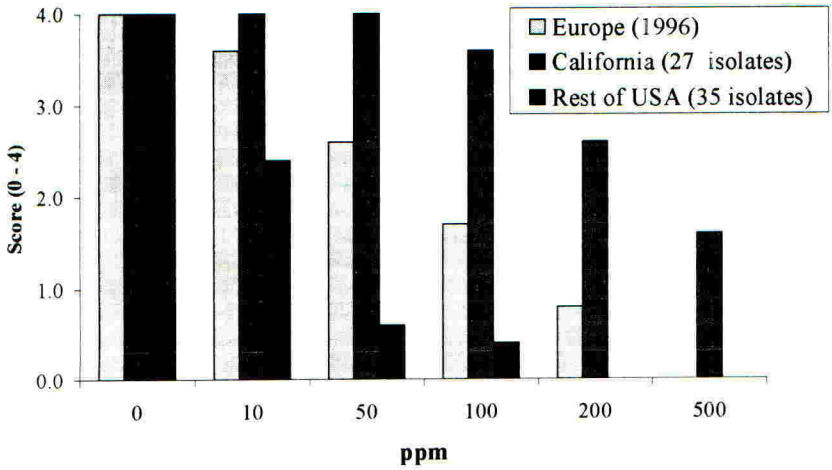


Figure 1. Median response of European *P.infestans* isolates to propamocarb for the years 1994–1997.

The data in Figure 1 show that sensitivity to propamocarb remained constant in 1994 and 1995. In 1996, there was a small increase in sensitivity to propamocarb, and this was followed by a highly significant increase in sensitivity ($P < 0.001$) in 1997. This large increase in sensitivity is difficult to account for, but may possibly be due to the advantageous weather for late blight of potato during this year selecting for faster growing isolates. These may be more effectively controlled by the cell membrane disrupting mode of action of propamocarb than slower growing isolates. Control isolates showed no significant variation in their response to propamocarb from year to year.

USA Isolates

The data for USA isolates are shown in Figure 2 and Tables 1, 2 & 3.



Rest of USA = North Dakota and surrounding states (20 isolates), New York (8 isolates) and Southern States (7 isolates)

Figure 2. Comparison of USA and European isolates median response to propamocarb.

Isolates from the Rest of USA were more sensitive than European isolates, whereas Californian isolates were less sensitive. At 100 ppm, the median value for European isolates was significantly different ($P < 0.001$) to both those from California and the Rest of USA. This result was surprising, as propamocarb has not been extensively used in California for the control of *P. infestans*. Any reduction in sensitivity to propamocarb might be expected in those states where propamocarb was being used routinely in late blight programmes. None of the Californian isolates were exceptional, when compared with the range of sensitivity seen with European isolates collected during the 5 years of the monitoring programme, and are within the normal distribution of European isolates sensitivities.

Isolates from California and the rest of the USA consisted of several races, with a high incidence of US8 (a predominantly phenylamide-insensitive, A2 mating type race) in both sample sets. There was no correlation in the sensitivity to propamocarb of US8 isolates from California and US8 isolates from the rest of USA.

Following these results it was decided to re-test four isolates from California which had scored >3 at 500ppm. The retest was carried out at the six concentrations used routinely, and an additional concentration of 1000 ppm was also included. In addition, these isolates were also tested for spore viability. This has previously been shown with European isolates to be the best discriminating test for determining propamocarb activity levels.

Table 1. Comparison of the sensitivity of Californian isolates to propamocarb – first test.

		Infection score						
		ppm						
Isolate	Year	0	10	50	100	200	500	1000
96.60.1	1996	4.0	4.0	2.4	3.0	0.8	0.0	NT
8548	1994	3.2	4.0	4.0	4.0	4.0	3.2	NT
8553	1994	3.2	4.0	4.0	4.0	4.0	3.4	NT
9461	1996	3.2	4.0	4.0	4.0	4.0	4.0	NT
9463	1996	4.0	4.0	4.0	4.0	4.0	4.0	NT

Table 2. Comparison of the sensitivity of Californian isolates to propamocarb – re-test.

		Infection score						
		ppm						
Isolate	Year	0	10	50	100	200	500	1000
96.60.1	1996	4.0	4.0	2.2	3.4	0.8	0.4	0.4
8548	1994	4.0	3.2	3.0	3.2	2.0	1.6	0.4
8553	1994	4.0	4.0	4.0	4.0	3.8	3.4	0.4
9461	1996	4.0	4.0	4.0	4.0	4.0	3.6	1.2
9463	1996	4.0	4.0	4.0	4.0	4.0	2.4	0.8

NT = Not tested.
Isolate 96.60.1 is a European isolate used as a reference control.

The data in Tables 1 and 2 show a good agreement, with the exception of Isolate 8548, which had a higher sensitivity in the re-test. However, it still remains an isolate with a lower sensitivity to propamocarb than the median value for USA isolates.

At 1000 ppm sporangiophore production was greatly reduced with all four isolates tested, and control of these isolates would be expected with a full field dose rate of propamocarb, which delivers between 2500 and 5000 ppm.

The mode of action of propamocarb (cell membrane disruption) causes it to have a variable EC_{50} depending on the life cycle stage of the pathogen, with the most potent activity seen against the formation of viable zoospores (Reich, 1994). Data from a test to determine the viability of the spores produced by the four less sensitive isolates are shown in Table 3.

Table 3. Viability of spores collected from four Californian isolates treated with propamocarb.

	Infection score		
	ppm		
Isolate	0	50	500
96.60.1	4.0	1.6	0.0
P8548	4.0	1.6	0.0
P8553	4.0	0.0	0.0
P9461	4.0	2.4	0.0
P9463	4.0	2.4	0.0

The data in Table 3 clearly show the good activity of propamocarb in preventing viable spore formation. Similar levels of activity were seen against the European isolate and the four isolates from California. Significant reduction in infection occurred at 50ppm, and all spores were non-viable at 500 ppm.

These results show that the isolates from California are as susceptible to propamocarb as other USA and European isolates. The most vulnerable life cycle stage, sporulation, is equally affected in all isolates. They would also indicate that the floating disk method of monitoring devised for phenylamide testing does not provide a complete answer for propamocarb sensitivity testing. Spore viability tests, which are more time-consuming and hence unsuitable as a routine monitoring method, need to be carried out before sensitivity levels can be fully evaluated.

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REFERENCES

Bardsley, R A; Shattock, R C; Day, J P (1996). Studies investigating the sensitivity of *Phytophthora infestans* to propamocarb hydrochloride. *Proceedings of the 1996 Brighton Crop Protection Conference – Pests and Diseases* **2**, 719-724.

Day, J P; Shattock, R C (1997). Aggressiveness and other factors relating to displacement of populations of *Phytophthora infestans* in England and Wales. *European Journal of Plant Pathology* **103**, 379-391.

Mann, H B; Whitney, D R (1947). *Annals of Mathematics and Statistics* **18**, 50.

Reich, B (1994). Zur wirkung von propamocarb-hydrochlorid auf *Phytophthora infestans* an kartoffeln und tomaten. *PhD thesis Hanover University*.

Secor, S A; Gudmestad, N C; Preston, D A; Rodriguez, J (1995). Late blight management in a traditionally blight-free area. *Proceedings of Phytophthora infestans 150*, 333-338

Sozzi, D; Schwinn, F J; Gisi, U (1992). Determination of the sensitivity of *Phytophthora infestans* to phenylamides: a leaf disk method. *EPPO Bulletin* **22**, 306-309.

Comparative studies on fungicide sensitivity and other characteristics in *Colletotrichum* isolated from various plant species

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ABSTRACT

Benzimidazole-resistant isolates of ripe rot fungus were frequently detected from infected grapevine, which had been treated with benomyl in dormant period. In the isolates, negative cross-resistance to diethofencarb was not observed. Comparative studies on fungicide sensitivity, conidial morphology, cultural characteristics, and PCR amplification of ITS regions of rDNA were carried out using a number of *Colletotrichum* isolates collected from various plant species. Most of benzimidazole-resistant isolates obtained from fruit tree showed resistance (insensitivity) to diethofencarb. In contrast, benzimidazole-resistant isolates of *C. gloeosporioides* (teleomorph: *Glomerella cingulata*) derived from strawberry exhibited negative cross-resistance to diethofencarb. It was shown that grapevine ripe rot is caused either by *G. cingulata* or *C. acutatum* and that the latter fungus is inherently resistant both to benzimidazoles and diethofencarb. A species-specific primer was used for the detection of *C. acutatum* from grapevine tissue naturally infected with ripe rot. Application of benzimidazole fungicides may increase populations of *C. acutatum* in vineyards.

INTRODUCTION

It was found that dormant spray applications of benomyl suppressed grapevine ripe rot (*Glomerella cingulata*) in subsequent growing seasons (Fukaya, 1996). Some years later, however, isolates of *Colletotrichum* showing resistance (insensitivity) to benomyl were frequently detected from infected grapevine in an experimental vineyard, which had previously been treated with the fungicide, although the performance of benomyl was still maintained in the field. Furthermore, the negative cross-resistance to diethofencarb was not seen in benzimidazole-resistant isolates thus obtained despite that those isolates were first regarded as *G. cingulata*, the teleomorph of *C. gloeosporioides* (Fukaya *et al.*, 1998). This character is remarkably different from that of *G. cingulata* isolated from other plants, e.g. strawberry.

To understand the taxonomic relationship of the pathogen causing ripe rot, comparative

studies on fungicide sensitivity, conidial morphology, cultural characteristics, and polymerase chain reaction (PCR) were carried out using a number of *Colletotrichum* isolates collected from various plant species within Japan.

METHODS AND MATERIALS

Fungal isolates and fungicides

Fifty-three isolates obtained from fruit trees (akebia, apple, blueberry, grapevine, Japanese apricot, Japanese chestnut, Japanese persimmon, loquat, peach, and prune), soft fruit (strawberry), vegetables (cucumber and watermelon), flowers (cyclamen) and woody plants (tea, locust-tree (*Robinia Pseudo-acacia*), and *Amorpha fruticosa*) were used. The benzimidazole fungicide benomyl (500 g/kg WP) was purchased and *N*-phenylcarbamate fungicide diethofencarb (250 g/kg WP) was a gift from Sumitomo Chemical Co. Ltd.

Tests for fungicide sensitivity

Each isolate was cultured on potato dextrose agar (PDA) plates at 25°C for five days. Mycelial discs, 4 mm in diameter, were cut from the margins of colonies and transferred onto PDA plates containing 0, 1, 10, and 100 mg/litre of a fungicide. Benomyl was added to PDA before autoclaving and diethofencarb was added to molten PDA after autoclaving. After incubation at 25°C for three days, the growth of mycelia was observed and the minimum inhibitory concentration (MIC) was determined for each fungicide. Resistance (insensitivity) or sensitivity of the isolates was distinguished as follows: resistant, growth at 100 mg/litre; sensitive, no growth at 100 mg/litre.

Conidial morphology and cultural characteristics

For morphological observations and measurements of conidia, isolates were grown on PDA plates under black light blue lamp 12 hr a day at 25°C. To determine morphology, conidia formed on PDA were mounted in water and observed using a light microscope. Fifty conidia of each isolate were measured in length and width. Colony colour was also examined approximately one month later after incubation on PDA.

Each isolate was cultured on PDA plates at 25°C for five days. Mycelial discs, 4 mm in diameter, were cut from the margins of colonies and transferred onto PDA plates. After seven days of incubation at 25°C, colony diameter was measured.

Extraction and purification of fungal DNA

Mycelia obtained from cultures grown on PDA plates at 25°C were used. Total DNA was extracted using the method of Adaskaveg & Hartin (1997).

Young fruit bunches (c. 10 mg fresh wt) of grapevine infected with ripe rot were boiled

in the extraction buffer (0.2M Tris-HCl, 0.25M NaCl, 25mM EDTA, and 2% sodium dodecyl sulphate, pH 8.5). Then total DNA was purified using 'QIAGEN DNeasy Plant Mini Kit' according to the manufacturer's protocol.

PCR experiments

Based on the published DNA sequences (Mills *et al.*, 1992; Sreenivasaprasad *et al.*, 1996), species-specific target primers were synthesized for *C. gloeosporioides* (CgInt) 5'-GGCCTCCCGCCTCCGGGCGG-3', and for *C. acutatum* (CaInt2) 5'-GGCGCCGGCCCCGTCACGGGGG-3'. Each target primer was used with the primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') from a conserved sequence of the fungal rDNA (White *et al.*, 1990). PCR reaction mixtures contained genomic DNA, buffer, 200 μM of each dNTP, 0.5 μM each of primers and 2.5 units of Taq DNA polymerase, and were subjected to 35 cycles of denaturing (1 min at 94°C), annealing (2 min at 59°C), and DNA extension (2 min at 72°C) on a thermal cycler. Following amplification, PCR products were analysed using electrophoresis on a 1.5 % agarose gel.

RESULTS

Response to fungicides

Based on the phenotypic difference of sensitivities to benomyl and diethofencarb, isolates were divided into three groups as follows: benomyl-sensitive and diethofencarb-resistant (S, R), benomyl-resistant and diethofencarb-sensitive (R, S), and resistant both to benomyl and diethofencarb (R, R). These three types were found in isolates from vegetables, flowers, and trees (Table 1). Interestingly, almost all of fruit tree isolates used in this study were either S, R or R, R type. Isolates donated as *C. acutatum* all showed the phenotype of R, R and this result confirmed earlier findings (Sato *et al.*, 1998).

Conidial morphology and cultural characteristics

As described before (Sutton, 1992), conidia of representative *C. gloeosporioides* are cylindrical with obtuse apex and most of the S, R and R, S isolates belong to this type. Conidia of typical *C. acutatum* are fusiform and each end of the conidium is abruptly tapered. The latter type was often found in the isolates showing resistance both to benomyl and diethofencarb (R, R).

When conidial size was compared, grapevine isolates fell in two groups as same as those from strawberry and small conidial group seemed to be *C. acutatum* (Table 1). However, even in the same isolate considerable variation was recognized in the size and shape of conidia.

Colony colour of the isolates varied markedly and included white, cream, dark brown, orange, or pink. There was no relationship between the colony colour and the host plants from which the isolates were obtained.

Similarly with the isolates from strawberry, both fast-growing and slow-growing isolates were collected from grapevine (Table 1). Slow growing isolates were also found in those from other fruit trees and woody plants such as locust-tree, naturally-growing near vineyards and orchards. However, characterization of species based on the growth speed seemed to be difficult since both types of isolates were found in *G. cingulata* (or *C. gloeosporioides*) isolates donated.

PCR analysis (Table 2)

When the primers CgInt and ITS4 were used, a DNA fragment of c. 450 base pairs (bp) was amplified with benomyl-sensitive isolates from grapevine and strawberry. In contrast, the primer CaInt2 in conjunction with ITS4 produced a fragment band of c. 500 bp with the isolates showing resistance both to benomyl and diethofencarb. Size of these DNA bands coincided well to that reported earlier for *C. gloeosporioides* and *C. acutatun* (Mills *et al.*, 1992; Sreenivasaprasad *et al.*, 1996).

A product of identical size (c. 450 bp) to that amplified from fungal pure cultures was produced when primers CaInt2 and ITS4 were used in PCR experiments with total DNA purified from infected grapevine tissue. In addition, the use of an allele-specific PCR primer has successfully amplified the fragment of ß-tubulin gene encoding resistance both to benomyl and diethofencarb (Ishii *et al.*, unpublished).

In conclusion, benomyl-sensitive isolates from grapevine ripe rot were determined as *C. gloeosporioides* and ones inherently resistant (insensitive) both to benomyl and diethofencarb as *C. acutatum*. Yamamoto *et al.* (1997) also isolated *C. acutatum* from grapevine and proposed to include this species in the pathogens of ripe rot. We support this idea but we also suspect that isolates of *C. acutatum* are widely distributed in fungal populations in vineyards and orchards.

Furthermore, it is likely that the application of benzimidazole fungicides increases populations of *C. acutatum*. Resistance monitoring will be necessary to be done as the mixture of diethofencarb with benzimidazoles is now used in practice.

Table 1. Fungicide sensitivity, conidial size, and mycelial growth of selected isolates of *Colletotrichum* spp. obtained from various plant species

Isolate	Host	Fungicide sensitivity*		Conidial size (μm)	Mycelial growth** (mm)
		Benomyl	Diethofencarb		
GC-2	Grapevine	S	R	12.7-15.8 x 4.3-5.7	72.1 ± 0.86
G5-1 7	Grapevine	R	R	9.6-15.2 x 3.5-5.1	58.7 ± 2.74
OHAF-1	Apple	R	R	10.4-15.7 x 3.8-5.7	60.2 ± 0.95
FPeCG9301	Peach	R	R	11.7-16.0 x 3.1-5.6	42.3 ± 1.82
NM-1	Strawberry	S	R	Not tested	74.9 ± 0.78
AL-9	Strawberry	R	R	13.3-17.4 x 4.1-5.8	40.3 ± 4.90
C3-P4Red	Strawberry	R	R	10.4-15.7 x 4.3-6.0	58.4 ± 1.08
270	Tea	R	S	11.8-17.1 x 3.6-5.5	45.8 ± 1.23
GC-RP-8	Locust-tree	R	R	13.2-21.0 x 3.9-6.2	57.6 ± 1.37
OILA-1	*A. fruticosa*	R	R	8.8-17.1 x 2.8-5.7	37.5 ± 5.02
(*C. gloeosporioides*:				12-17 x 3.5-6)***	
(*C. acutatum*:				8.5-16.5 x 2.5-4)***	

*S, sensitive; R, resistant (insensitive).

**Mean ± SD of colony diameter seven days after incubation.

***Values from Sutton (1992).

Table 2. PCR amplification of ITS regions of rDNA for isolates of *Colletotrichum gloeosporioides* and *C. acutatum*

Isolate	Fungicide sensitivity	Primer with ITS4*		Evaluation
		CgInt	CaInt2	
Grapevine ripe rot:				
GC-2	S, R	+	–	*C. gloeosporiodes*
G1-1 5	R, R	–	+	*C. acutatum*
G5-1 7	R, R	–	+	*C. acutatum*
Strawberry anthracnose:				
NM-1	S, R	+	–	*C. gloeosporiodes*
Na91-016	R, R	–	+	*C. acutatum*

*+, DNA amplified; –, DNA not amplified.

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REFERENCES

Adaskaveg J E; Hartin R J (1997). Characterization of *Colletotrichum acutatum* isolates causing anthracnose of almond and peach in California. *Phytopathology* **87**, 979-987.

Fukaya M (1996). Control of grapevine ripe rot in dormant period. *Annual Report of the Society of Plant Protection of North Japan* **47**, 161.

Fukaya M; Ishii H; Takahashi I (1998). Dormant spray of benomyl for the control of grape ripe rot and detection of fungicide-resistant isolates of the fungus. In: *Abstracts of Annual Meeting of the Phytopathological Society of Japan*, p. 137.

Mills P R; Sreenivasaprasad S; Brown A E (1992). Detection and differentiation of *Colletotrichum gloeosporioides* isolates using PCR. *FEMS Microbiology Letters* **98**, 137-144.

Sato T; Uematsu S; Iijima A; Koganezawa H (1998). Occurrence of apple bitter rot by grayish colony form of *Colletotrichum acutatum* in Japan and pathogenicity to apple fruits of *C. acutatum* and *Glomerella cingulata* isolated from other plants. *Nippon Kingakukai Kaiho* **39**, 35-44.

Sreenivasaprasad S; Sharada K; Brown A E; Mills P R (1996). PCR-based detection of *Colletotrichum acutatum* on strawberry. *Plant Pathology* **45**, 650-655.

Sutton B C (1992) The Genus *Glomerella* and its anamorph *Colletotrichum*. In: *Colletotrichum: Biology, Pathology and Control*, eds. J A Bailey & M J Jeger, pp. 1-26. C·A·B International: Wallingford, UK.

White T J; Bruns T; Lee S; Taylor J (1990). A guide to methods and applications. In: *PCR Protocols*, eds. M A Innis, D H Gelfand, J J Shinsky & T J White, pp. 315-322. Academic Press: San Diego. USA.

Yamamoto J; Sato T; Tomioka K (1997). Ripe rot of grapes (*Vitis vinifera* Linn.) caused by *Colletotrichum acutatum*. *Annals of the Phytopathological Society of Japan* **63**, 527.

Factors affecting strength of selection for resistance to DMI fungicides in *Septoria tritici*

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ABSTRACT

The effects of dose and fungicide systemicity on selection for resistance were examined for prochloraz, flutriafol and fluquinconazole. In an inoculated field experiment using 1/8 or 1/4 field doses, sprays of all fungicides caused significant selection for resistance. The strength of selection varied with fungicide, position of infection in the canopy and along individual leaves. Increasing dose from 1/8 to 1/4 and applying split 1/8 doses significantly increased selection. In glasshouse experiments, fungicide application caused unexpected stimulation of infection and selection for susceptibility. Tracing of radio-labelled fluquinconazole showed that translocation was modified by infection.

INTRODUCTION

Sterol 14 α demethylation inhibitor (DMI) fungicides are very effective for control of *S. tritici* and are used extensively, with at least one application per year in most wheat crops in the UK. Field resistance to DMI fungicides is known to have occurred in *Erysiphe graminis* populations and is a potential risk with *S. tritici*. There is a lack of understanding of the dynamics of resistance development to fungicides and the many interacting factors that could influence the short-and long-term survival of resistant fungal isolates in field populations. The general aims of this work were to identify factors which could influence the strength of selection of resistance.

There is much controversy over whether reducing doses or applying split applications of fungicides can affect the strength of selection for resistance. Some DMI s are highly systemic, while others are non-translocated. Compounds contrasting in mobility will accumulate in different parts of leaves. This has been shown to affect disease severity (Shephard, 1985) and could influence strength of selection. This paper describes a series of glasshouse and field experiments designed to determine (1) how strength of selection is affected by dose and split applications, (2) whether strength of selection varies along individual leaves and (3) whether physio-chemical properties of DMI fungicides can influence strength of selection.

MATERIALS AND METHODS

In all experiments, wheat plants were inoculated with *S. tritici* isolates of known fungicide sensitivity. A total of four isolates of similar pathogenicity were used, two from each extreme of the flutriafol sensitivity spectrum. Unless otherwise stated inoculum concentrations were

2×10^5 conidia/ml. Flutriafol ('Impact', Zeneca plc.) and fluquinconazole ('CR21484'; AgrEvo Ltd) were chosen for their systemic activity. Prochloraz ('Sportak', AgrEvo Ltd), a non-translocated compound, was chosen to contrast with flutriafol and fluquinconazole.

Field experiment

In 1997, field plots of spring wheat cv. Baldus were spray inoculated at GS 13 with 2×10^6 conidia/ml of equal proportions of each isolate at a rate of 1500 litres water/ha. All fungicides were sprayed at either 1/8, 1/4 or 2 x 1/8 recommended field doses at GS 39-47, corresponding to flutriafol 15.6 g/ha, or 31.25 g/ha, fluquinconazole 18.75 g/ha or 37.5 g/ha and prochloraz 50 g/ha, or 100 g/ha. Plots receiving 2 x 1/8 were sprayed 14 days later at GS 65-69 with a further 1/8 dose. Plots sprayed with water plus 'Tween 80' (0.01% v/v) served as standards for comparison of treatments. Selection for resistance was measured using an *in vitro* discriminating dose assay based on a previously described micotitre plate assay (Pijls *et al*, 1994) to determine the difference between the ratio of susceptible to resistant isolates in each field plot before and after fungicide application. A total of 5130 isolates were sampled. The strength of selection was compared for each treatment on each of the top two leaves of the crop canopy.

Glasshouse experiments

In experiment one, single 1 µl droplets of either fungicide were applied to the second and third leaves of wheat seedlings cv. Riband at GS 13. Flutriafol (0.125 g/litre or 0.25 g/litre), fluquinconazole (0.15 g/litre or 0.3 g/litre) or prochloraz (0.4 g/litre or 0.8 g/litre) were applied at either 30 mm or 5 mm above or below the site of inoculation. The same leaves were inoculated with either a resistant or susceptible isolate applied as single 2 µl droplets in the middle of each leaf. Inoculated plants not treated with fungicide were included to compare treatments. 30 replicated leaves were included per treatment. The number of pycnidial lesions at 24 days post inoculation (dpi) was compared for each treatment.

In experiment two, sprays of flutriafol (15.6 g/ha, or 31.25 g/ha), fluquinconazole (18.75 g/ha or 37.5 g/ha) or prochloraz (50 g/ha, or 100 g/ha) were applied to wheat seedlings of cv. Riband at GS 13. Plants were inoculated with either a resistant or susceptible isolate one week after application. Presence of pycnidial lesions was recorded at 2 cm intervals along the length of leaves 2 and 3. Two measures were calculated for each position. First was *control*: the difference between the frequency of lesions on fungicide treated and untreated leaves, divided by the standard error of the difference. Second was *selection*: the difference between isolates in the degree of control. Results for flutriafol are presented.

Radio-tracing

In a growth chamber experiment, single 1 µl droplets of a ^{14}C radio-labelled fluquinconazole formulation containing (0.3 g a.i./litre) were dispensed to the middle of wheat leaves cv. Riband at GS 13. The same leaves were inoculated with 1 µl droplets of either resistant or susceptible isolates applied 30 mm above the fungicide application sites. Fungicide treated, but uninoculated leaves were included for comparison. At 1, 6, and 15 DAT leaves were excised and the radioactivity detected at 1 mm intervals along each leaf using a radio thin layer chromatography analyser ('RITA 3200').

RESULTS

Averaged over leaves, the best control in the field was provided by fluquinconazole, followed by flutriafol, then prochloraz (not presented). Selection by flutriafol and prochloraz was similar on average; selection by fluquinconazole was somewhat stronger (Figure 1). Fluquinconazole and flutriafol selected less strongly at the base of leaves. Averaged over all fungicides on leaf two the 1/4 doses selected significantly more than 1/8 or 2 x 1/8 doses (Figure 2). On leaf one 2 x 1/8 doses of fungicides selected significantly more than any of the single applications.

In glasshouse experiment one, application of fungicides caused significant control of *S. tritici*. (Figure 3). Averaged over all positions, all fungicides caused significant selection for resistance (not presented), but there was no significant difference between strength of selection caused by each fungicide. Flutriafol caused significant selection when applied 30 mm above and below inoculation sites, but fluquinconazole only caused significant selection when applied 30 mm above inoculation sites (Figure 3). In contrast, prochloraz did not cause significant selection at any single position. Dose rates had no significant effect on strength of selection (data not presented).

In glasshouse experiment one, no selection occurred where control was either very high (fungicide position, -5 mm) or very low (fungicide position, +5 mm) because this prevented any differential effects on the isolates. Similarly in experiment two, fluquinconazole provided the best control, but caused the least selection (not presented).

In glasshouse experiment two, control and selection for resistance occurred most strongly at leaf tips on plants sprayed with flutriafol (Figure 4). There were areas of leaves where the application of fungicides stimulated infection by both isolates. Strength of selection for resistance increased with time from 17 to 27 dpi. Selection for susceptibility was also observed, though this decreased with time. Control and selection by prochloraz and fluquinconazole were more evenly distributed along leaves.

In the radio-tracing studies, fluquinconazole was translocated acropetally from the middle of leaves. In inoculated leaves, fluquinconazole accumulated at inoculation sites from 6 dpi (Figure 5) until leaf senescence at 20 dpi (not presented).

DISCUSSION

Application of fungicides resulted in significant selection for resistance (at the reduced doses used) in all field and glasshouse experiments. These results are consistent with other inoculated field experiments (Peever *et al.*, 1994). However, significant shifts in sensitivity have not been measured in naturally occurring populations of *S. tritici* treated with DMI s (Pijls & Shaw, 1997). There have been indications that reduced doses could speed up resistance development (Engels & De Waard, 1994). We present evidence that decreasing dose from 1/4 to 1/8 can decrease selection, which is consistent with other work on *S. tritici* (Porras *et al.*, 1990) and barley powdery mildew (Hunter *et al.*, 1984). However, split applications did significantly increase selection probably by maintaining selection pressure over a longer period. Flutriafol and fluquinconazole caused most selection at leaf tips. This is explained by translocated compounds accumulating acropetally and thus causing higher selection pressure at leaf tips.

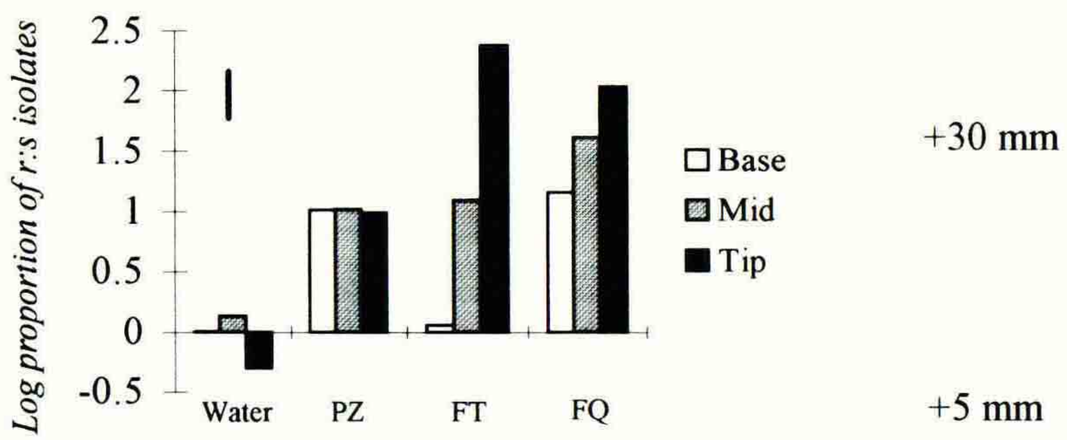

Figure 1. Strength of selection along leaves sprayed with water, prochloraz (PZ), flutriafol (FT) and fluquinconazole (FQ). Bar indicates LSD at 5%.

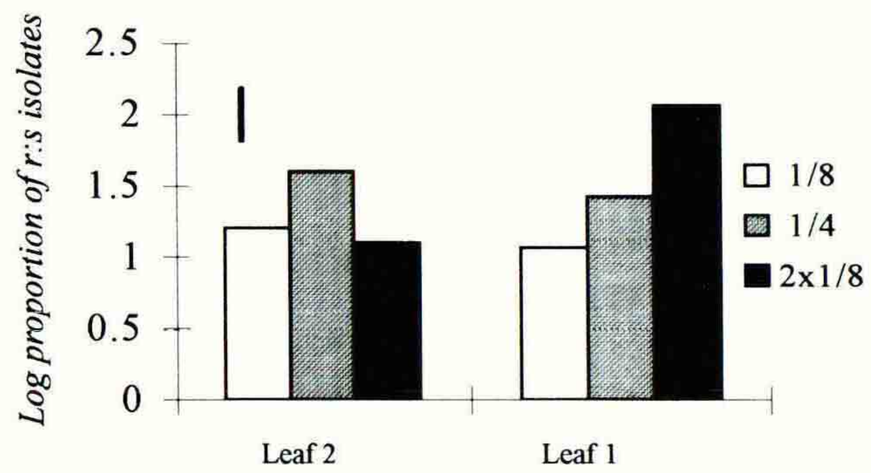

Figure 2. Effect of fungicide dose on strength of selection on the top two leaves of the crop canopy. Bar indicates LSD at 5%.

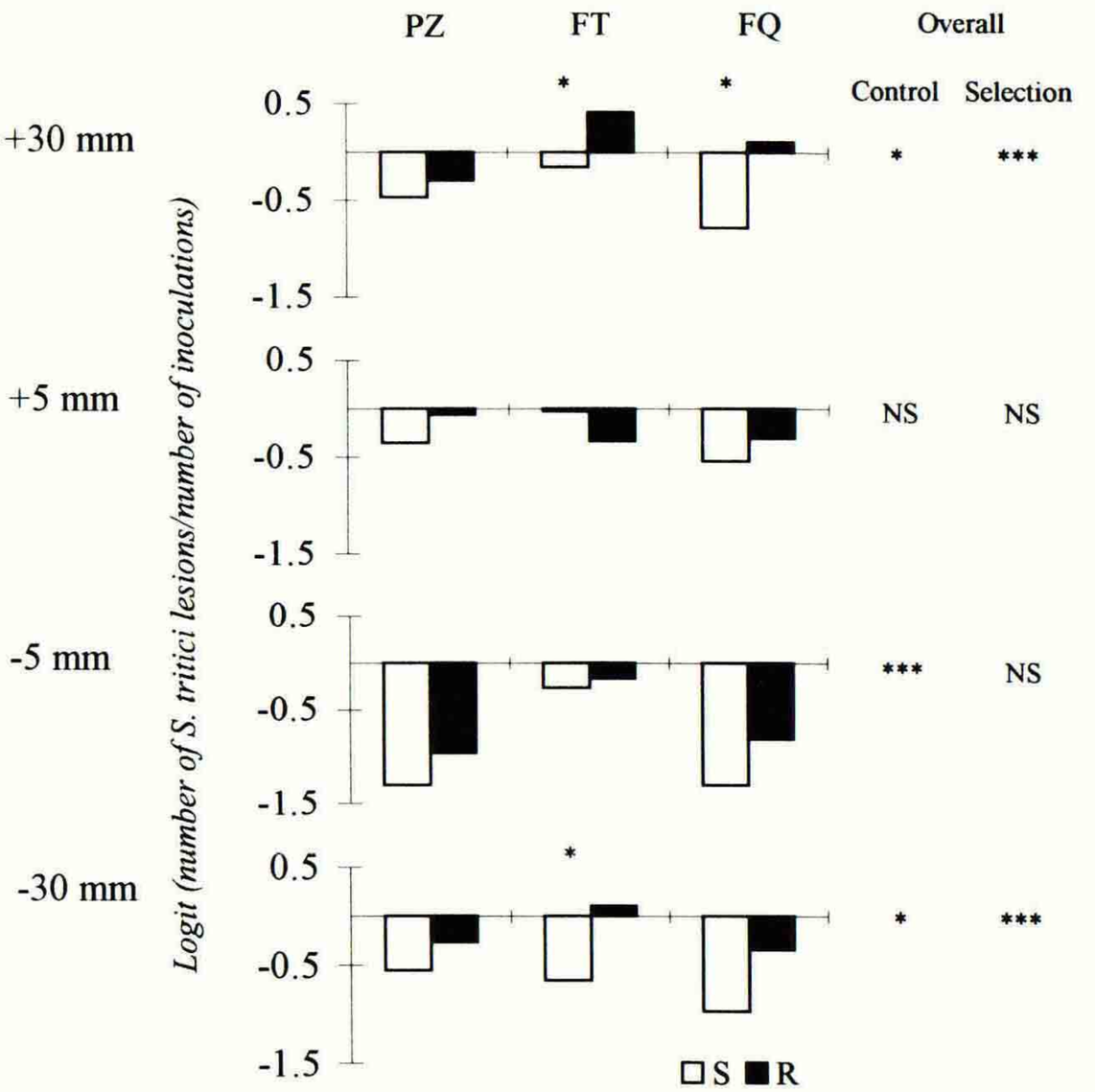

Figure 3. Control of *S. tritici* and selection for resistance caused by prochloraz (PZ), flutriafol (FT) and fluquinconazole (FQ) applied at different distances from inoculation sites. Significance is indicated by: * $P = 5\%$; *** $P = 0.1\%$; NS not significant.

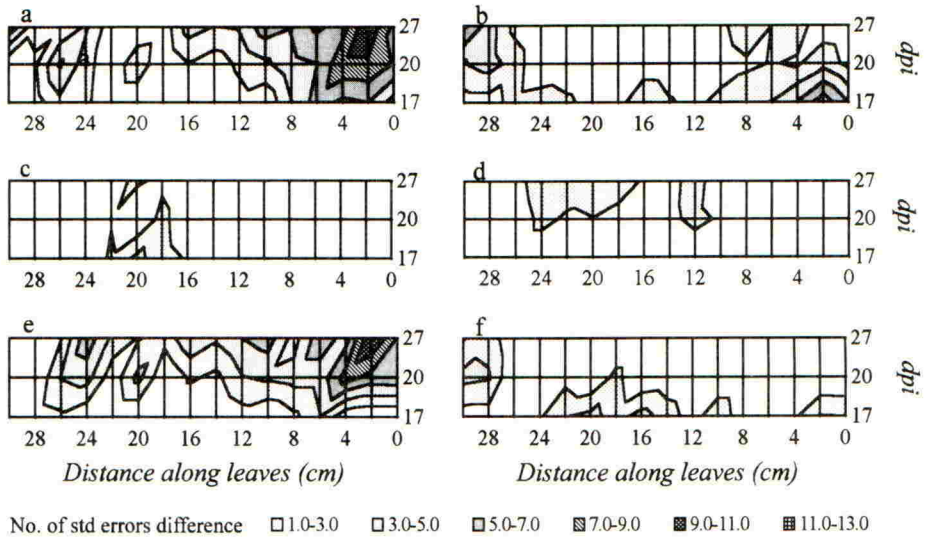

Figure 4. Effects of flutriafol sprays on (a) control of susceptible isolates; (b) control of resistant isolate; (c) stimulation of susceptible isolate; (d) stimulation of resitstant isolate; (e) selection for resistance; (f) selection for susceptibility. 0 cm is leaf tip.

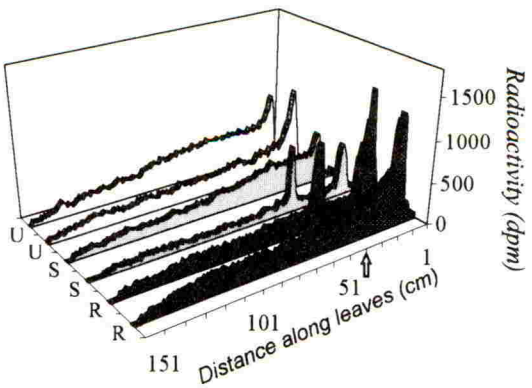

Figure 5. Translocation of ^{14}C radiolabelled fluquinconazole in wheat seedling leaves at 6 DAT either uninoculated (U) or inoculated with susceptible (S) or resistant (R) isolates of *S. tritici* applied at 30 mm above fungicide sites; inoculation point indicated by (⇧).

In contrast, non-translocated prochloraz caused even selection along leaves. There was evidence that the rapid mobility of flutriafol could reduce overall selection by moving away from basal areas of leaves.

Application of fungicides caused stimulation of infection in both glasshouse experiments. A number of diseases which result from or increase in severity because of application of crop protection chemicals have been reported (Griffiths, 1981). If isolates contrasting in fungicide sensitivity are stimulated to different degrees, this could affect strength of selection. In

glasshouse experiment two, stimulation of the resistant isolate caused significant selection to occur in the + 30 mm treatment. Radio-tracing studies have shown that fluquinconazole is only translocated acropetally (not presented), suggesting this effect was the result of a host response.

Microscopical studies have shown that the resistant isolate produced significantly more surface and internal mycelium during latency than the susceptible isolate (not presented). This may have caused the resistant isolate to come in to contact with more fungicide than the susceptible isolate early in the infection process, resulting in the selection for susceptibility in experiment two.

Clearly, infection by *S. tritici* can modify translocation of fluquinconazole. Peaks were present from 6 dpi. At this time initial penetration of host leaves was occurring and transpiration rates at infection sites were significantly higher than in uninfected leaves (not presented). This may suggest fluquinconazole accumulation is due to a host response to infection. This phenomenon could either increase strength of selection by increasing selection pressure around infection sites or reduce selection by increasing control and preventing any differential effects on resistant and susceptible isolates.

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REFERENCES

Engels A J G; De Waard M A (1994). Effects of different treatment schedules on the sensitivity of *Erysiphe graminis* f.sp. *tritici* to fenpropimorph. In: *Fungicide Resistance*. BSPP Monograph No. 60, ed. S Heaney, D Slawson, D W Hollomon, M Smith, P E Russell, D W Parry. British Crop Protection Council: Farnham.

Griffiths E (1981). Iatrogenic plant diseases. *Annual Review of Phytopathology* **19**, 69-82.

Hunter T; Brent K J; Carter G A (1984). Effects of fungicide regimes on sensitivity and control of barley mildew. *British Crop Protection Conference - Pests & Diseases*, 471-476.

Peever T L; Brants A; Bergstrom G C; Milgroom M G (1994). Selection for decreased sensitivity to propiconazole in experimental field populations of *Stagonospora nodorum* (syn. *Septoria nodorum*). *Canadian Journal of Plant Pathology* **16**, 109-117.

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

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.

Porras L; Gisi U; Staehle-Csech U (1990). Selection dynamics in triazole populations of *Erysiphie graminis* on barley. *Brighton Crop Protection Conference - Pests & Diseases,* 1163-1168.

Shephard M C (1985). Fungicide behaviour in the plant : systemicity. *British Crop Protection Council Monograph* **31,** 99-106.

POSTER SESSION 6D

MANAGEMENT OF PESTS AND DISEASES IN TROPICAL CROPS

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Poster Papers

6D - 1 to 6D - 5

MATERIALS AND METHODS

Insect culture: *S. oryzae* strains from India and from Sri Lanka were cultured in glass jars on organic brown rice (U-Natura, Italy) under controlled temperature and humidity (CTH; 28°C and 58% R.H.), with a 12 hours light cycle.
Plant material:- Dried *Gardenia fosbergii* leaf bud exudate (Tamil name: Lakede), was obtained from a local ayurvedic shop in Batticaloa, Sri Lanka. The exudate was ground, extracted in methanol overnight and then evaporated *in vacuo* in a rotary evaporator. The dried extract was redissolved in acetone to obtain 100 and 1000 mg/l extracts. Seeds of *Azadirachta indica* from Bvumbwe, Malawi, were ground and extracted as described above for *Gardenia* using the same concentations.
Organic brown rice of the same variety used for the insect cultures was conditioned to 14.5% moisture content (determined by oven-drying at 130°C for 45 min) by keeping it under CTH conditions seven days before the start of the experiment. It was then coated with *Gardenia* and Neem extracts at either 100 or 1000 mg/l (treatments), by soaking it for 10 sec. in the extract and then pouring off the extract. Subsequently the rice was completely dried in a fume cupboard. Rice coated with acetone was used as a control.
Bioassay: Two choice-tests were set up in three-way petridishes of 9 cm diameter using the Indian insect strain in a first test and the Sri Lankan strain in a second test. Three grams of treated rice were placed in compartment 1, control rice (3 g) was placed in compartment 2 and eight insects aged between 1-7 days were released into the third empty compartment. The experiment was carried out in a CTH room. Each treatment was replicated 10 times. The number of insects in each compartment was recorded after 7 d and 28 d.
HPLC analyses: Extracts kept at -20°C were analysed using a Waters system (Waters 600E pump, Waters™996 PDA Detector at 200-400nm) and a Lichrospher 100 RP 18 column (25cm x 4mm i.d., Merck) with a 5µm particle size. The mobile phase consisted of a solvent gradient of 25/75 acetonitrile/water (with 1% acetic acid added) and a flow rate of 1ml/min at time 0 min, reaching 100% acetonitrile after 25 min.
To determine if inactivation of *Gardenia* extracts was possibly influenced by daylight, one extract was kept in the daylight for 3 days and compared to an extract which had been kept in the dark and in the freezer at -20°C.

RESULTS AND DISCUSSION

In the first experiment *S. oryzae* adults were strongly deterred from feeding on *Gardenia* treated rice and this effect was highly significant ($p = 0.001$ for the 1000 mg/l treatment and $p<0.01$ for the 100 mg/l treatment, using t-Test). Seven days after being presented with a choice between solvent treated and *Gardenia* leaf bud exudate treated rice, 98% of insects in the 1000 mg/l treatment and 78% in the 100 mg/l treatment were feeding on control rice (Fig. 1). After 28 days nearly 100% of insects were feeding on control rice in both treatments.

A similar effect was observed for insects presented with rice treated with Neem extracts, but the deterrent effect was not as potent as that observed for the *Gardenia* extracts at the same concentrations (Fig. 1).
The bioassay has demonstrated that *Gardenia* leaf bud exudate contains compounds which are strongly deterrent to adults of *S. oryzae* and that their effect is concentration dependent.

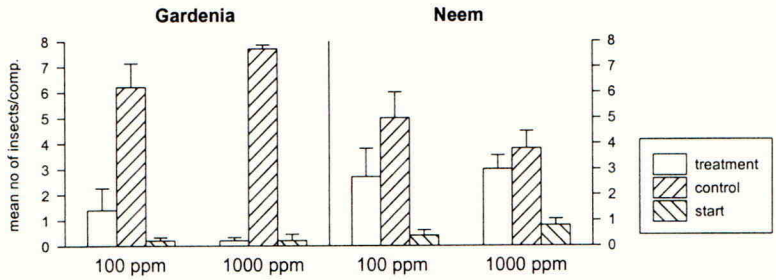

Figure 1. No. of *S. oryzae* adults (Indian strain) in each compartment of petridish after 7 days of choice between control rice and rice treated with *Gardenia* or Neem seed extracts (treatment).

In the second bioassay (Sri Lankan strain of *S. oryzae*) no preference was observed for either the control rice or the rice treated with *Gardenia* extracts at either 100 or 1000 mg/l after 7 days of choice (Fig. 2).

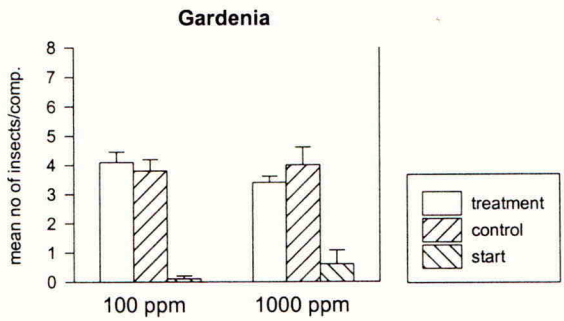

Figure 2. No. of *S. oryzae* adults (Sri Lankan strain) in each compartment of petridish after 7 days of choice between control rice and rice treated with *Gardenia* extracts (treatment).

HPLC analyses of *Gardenia* leaf bud exudates revealed differences in chemical profiles between deterrent extracts, used inthe first test, and inactive ones, used in the second experiment, suggesting an association of several compounds with the activity or inactivity of the extracts. There was also a difference between chromatograms of extracts kept in the daylight and in the dark, obtained at 210 nm (Fig. 3). The extract kept in the dark contained several compounds (peaks B, E, F, G in the chromatogram) which were absent in the extract exposed to light, suggesting they are degraded and inactivated by light.

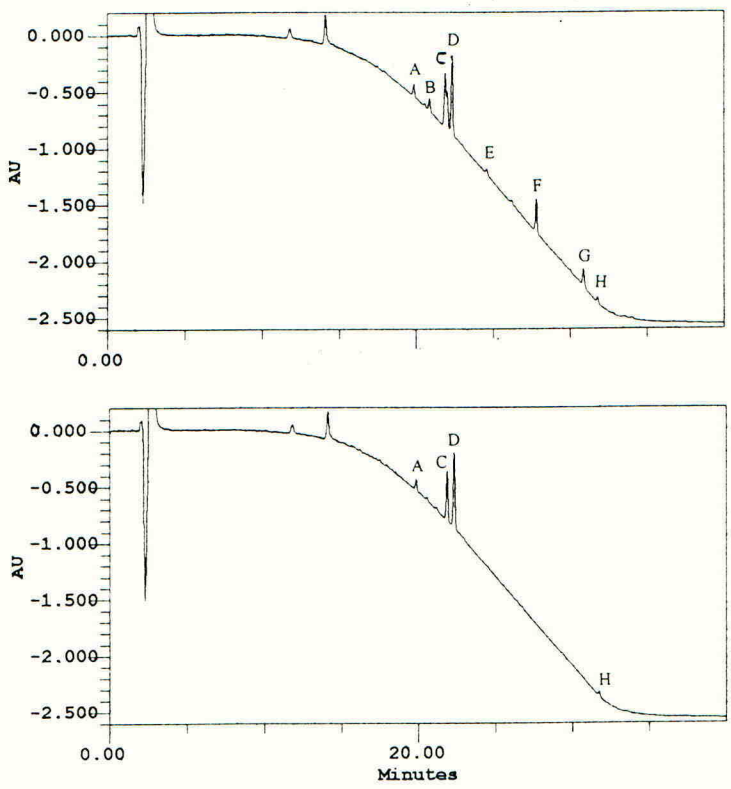

Figure 3. HPLC chromatograms of extracts of *Gardenia* kept in the dark (top) and in the daylight (bottom) obtained at 210 nm. Peaks B, E, F and G are missing in the light-exposed extract.

Chromatograms of the deterrent and inactive extracts of *Gardenia* leaf bud exudate used in the choice tests revealed a very similar chemical profile to the extracts kept in the dark and daylight conditions respectively (Fig. 4).

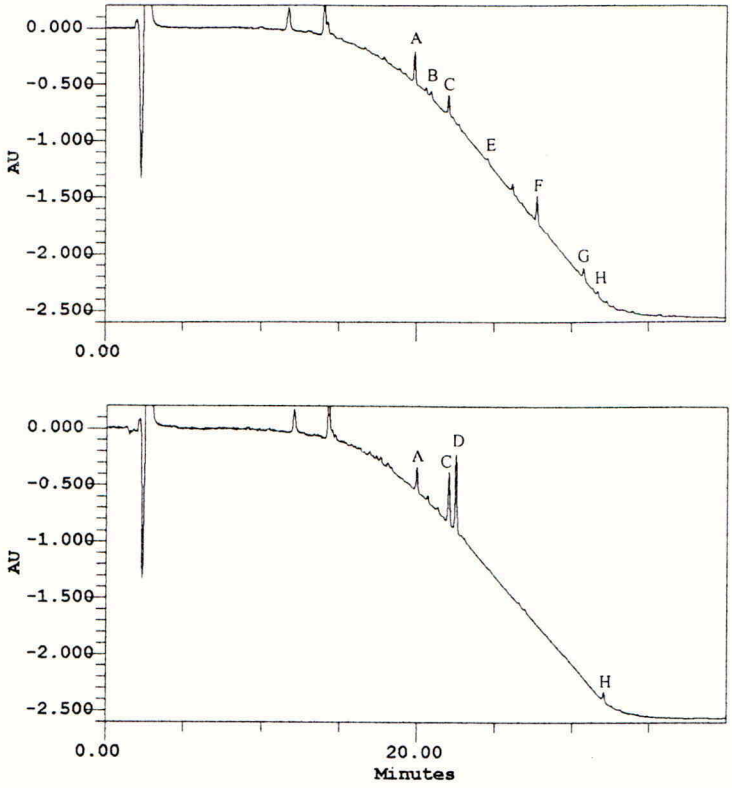

Figure 4. HPLC chromatograms of active Gar*denia* extract (top) and inactive extract (bottom) obtained at 210 nm.
Peaks B, E, F and G are missing in the inactive extract.

These results indicate that at least four compounds present in the extracts of *G. fosbergii* (peaks B, E, F and G) are associated with insect deterrent activity, and that their degradation may be caused by exposure to light.
These compounds have yet to be identified and their individual activity tested with further bioassays.
Gardenia fosbergii has the potential to be very valuable as a storage protectant because a possible degradation of its biologically active compounds by light would imply the possibility of overcoming toxic effects caused by the plant material by simply exposing the grains to direct sunlight, provided they do not break down into other toxic compounds.

It is also of interest to evaluate the potential of other *Gardenia* species, e.g. *G. cramerii*, another indigenous plant of Sri Lanka (Gunatilaka et al. 1982), or *G.*

jasmonoides, already known for its medicinal properties associated with flavonoids and terpenoids (Iida et al. 1991, Quin & Xu 1991).

This project is supported by scientists at Eastern University, Sri Lanka, as an opportunity to begin investigating local plant species which could be developed as pest control agents. By this example the interest in studying and retaining the indigenous biodiversity in Sri Lanka should be renewed, particularly in the eastern region which has suffered over the past 10 years from civil and military unrest.

REFERENCES

Golob P and Webley DJ (1980). The use of plants and minerals as traditional protectants of stored products, Report of the Tropical Products Institute, G138, vi

Gunatilaka AAL, Sirimanne SR, Sotheeswaran S and Sriyani HTB (1982). Flavonoids of *Gardenia cramerii* and *G. fosbergii* bud exudates. Phytochemistry **21**(3), 805-806.

Iida J, Ono M, Inoue K and Fujita T (1991). Thermospray liquid Chromatographic/ Mass Spectrometric analysis of iridoid Glycosides from *Gardenia jasminoides*. Chemical and Pharmaceutical Bulletin **39**(8), 2057-2062.

Longstaff BC (1981). Biology of the grain pest species of the genus *Sitophilus* (Coleoptera: Curculionidae): a critical review. Protection Ecology **2**, 83-183.

Qin G and Xu R (1991). Studies on the chemical constituents of *Gardenia jasminoides*. Planta Medica **57**(2), A75.

Strassburger E, Noll F, Schenck H and Schimper AFW, eds. Lehrbuch der Botanik, p 909. Gustav Vischer Verlag, Stuttgart, New York, 1983.

Development of biological control methods for post-harvest rots of banana

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ABSTRACT

Both treated and untreated banana fruit from different banana growing areaswere used as a source of inoculum to establish a collection of over one hundred and fifty pathogenic fungal isolates. These were identified as far as possible and the frequency of occurrence of the main crown rot pathogens, from the different areas, recorded. Attempts were made to establish the level of pathogenicity of the most commonly isolated fungi, and the sensitivity of some pathogens to the fungicide thiobendazole (TBZ) was assessed. Putative biocontrol agents were also isolated from banana material and their potential for control determined using a previously developed bioassay.

INTRODUCTION

Crown rot is one of the most severe post-harvest diseases of banana world-wide. It is caused by a complex of fungal pathogens of which *Colletotrichum* spp. and *Fusarium* spp. are regarded as the most significant. These attack the cut surface of the crown after harvest causing a soft rot to develop which results in a blackening of the crown tissue and often visible surface mould. The rot can proliferate throughout the crown and basal part of the fruit during ripening and so attack individual fingers, thus lowering the commercial value of the produce. The same pathogen complex also causes anthracnose on banana peel, a disease which also occurs world-wide, but is reported as severe in some areas.

Despite large-scale post-harvest dipping or spraying with fungicide, most of the commercial banana centres with an export component still encounter high crown rot levels and often unacceptable losses. This may be due to a combination of factors, including tolerance of the fungi to the fungicides used (Johanson & Blasquez, 1992), and the inaccessibility of latent fungal structures to the fungicides (Jeger & Jeffries, 1988). Combine this with an increasing market pressure for 'organic bananas' and stricter consumer protection standards and it is clear that alternatives to chemical control of both crown rot and anthracnose should be sought before the situation becomes irrecoverable.

There is strong incentive for developing successful biological control of crown rot for three main reasons:

1) Exact storage conditions can be maintained to suit the needs of the selected biocontrol agent.
2) The target area of harvested product is both well defined and less than that of the standing crop, therefore treatment costs can be reduced.
3) Any expenditure on control can be justified due to the high market value of the harvested produce (Wilson & Pusey, 1985).

Mycoparasites, commonly defined as fungi that parasitise other fungi, along with competitors and antagonists are all promising candidates for post-harvest application. It is understood that there has never been a report of a fungal host strain acquiring resistance to its mycoparasites, and so this may prove to be a sustainable control method when fungicides are losing their effectiveness.

MATERIALS AND METHODS

Isolation of fungi from infected crowns

Banana hands showing crown rot symptoms were obtained from JP Fruits, Dartford, Kent or from local supermarkets. The affected crown and pedicel of each hand was swabbed with 70% ethanol, allowed to dry and small pieces of tissue (four from each area) were asceptically removed from the edge of the rot. Samples were also taken from the 'flower end' and peel of infected fingers. All samples were placed on tap water agar at 26°C ± 2°C for 1-3 days. Subcultures from any visible fungal mycelia were made on potato dextrose agar and further incubated for identification purposes.

Pathogenicity testing of fungi isolated from banana crowns

Twelve fungal isolates were tested for their pathogenicity to banana tissue using a specifically developed bioassay (Krauss, 1996). The samples chosen were a cross-section of those isolated previously. Peel disks were cut from clean green fruit using a sterile cork borer (1cm diameter), soaked for 10 seconds in 10% sodium hypochlorite and then washed in sterile distilled water. Five replicate disks for each treatment were then soaked in spore suspensions of the test fungi, 10^4 spores ml^{-1} being used for *Colletotrichum* spp. and 10^5 spores ml^{-1} for *Fusarium* spp., *C. musae* having previously been reported to be twenty times more pathogenic than *Fusarium* spp. (Finlay, 1991). The disks were then placed in Petri dishes with moist filter paper in the lids and incubated at 26°C for several days. The percentage of each disk surface that had become black and rotted was recorded daily. This was assessed by eye using a photographic scale for comparison.

Screening for fungicide sensitivity

A stock solution of thiobendazole (45% w/v a.i) was incorporated into batches of PDA to yield six final concentrations, 0.01, 0.1, 1.0, 10, 100 and 1000µg/ml. The fungicide, suspended in ethanol, was added to the agar prior to autoclaving. Once sterilised the amended medium was poured into 50mm Petri dishes. A sterile cork borer was then used to cut mycelial plugs (4mm diameter) from the margin of a 7-day old pure culture. One plug was placed at the edge of each prepared plate. Three replicates were prepared for each TBZ concentration along with a control plate containing no TBZ but the corresponding amount of ethanol. Cultures were then incubated at 26°C, 12h light / 12h dark. Radial growth of each colony was measured from the edge of the mycelial plug 10 days after inoculation. Measurements of colony radius were converted into percentage inhibition of growth with respect to the control treatment (no fungicide). The concentration of fungicide that inhibited fungal growth by 50% (ED_{50}) was calculated.

Isolation of putative biocontrol agents

Plates precolonised with either *Colletotrichum musae* or *Fusarium* sp. were used to detect and subsequently isolate presumptive mycoparasites from both fresh green and dry banana leaves. Isolations were also made from banana peel disks which, having been dipped in a fungal suspension of known pathogenicity, had failed to show symptoms of rot, indicating the possible presence of competitors and/or antagonists. The potential of isolates to control a pathogenic strain of *C. musae* was then assessed using the previously described peel disk bioassay and results compared to those for TBZ applied to similar disks at a concentration of 10μg /ml (known ED_{50} of pathogen < 1μg /ml).

RESULTS

Isolation of fungi from infected crowns

Table 1. Frequency of isolation of different fungal species from infected crowns of banana imported into the UK

Fungus	Place of Origin			
	Jamaica	Windward Islands	Costa Rica	Dominican republic *
Colletotrichum musae	4 (5.1%)	2 (5.7%)	1 (3.3%)	8 (15.1%)
Colletotricum spp.	16 (20.3%)	8 (22.1%)	6 (20%)	12 (22.6%)
Fusarium spp.	21 (26.6%)	6 (17.7%)	6 (20%)	26 (49.1%)
Botryodiplodia theobromae	7 (8.9%)	2 (5.7%)	2 (6.6%)	0
Curvularia sp.	11 (13.9%)	0	2 (6.6%)	0
Nigrospora sphaerica	3 (3.8%)	0	1 (3.3%)	0
Penicillium	0	1 (2.9)	0	0
Cladosporium spp.	0	2 (5.7%)	0	0
Trichoderma sp.	0	0	2 (6.6%)	0
Aspergillus sp.	1 (1.3%)	0	0	0
Unknown fungi a**	7 (8.9%)	7 (20%)	4 (13.3%)	3 (5.7%)
Unknown fungi b	5 (6.3%)	5 (14.3%)	3 (10%)	2 (3.8%)
Unknown bacteria	4 (5.1%)	2 (5.7%)	3 (10%)	2 (3.8%)
Total isolated	79	35	30	53
No. of sample hands	5	2	4	2

* Fruit labelled as 'Organic' and marketed as untreated

** Fungi in this category, although unidentified, all had a similar morphology and are believed to be of the same species

Pathogenicity testing of fungi isolated from banana crowns

Table 2. Degree of rotting of banana fruit peel disks caused by fungal strains isolated from diseased banana fruit (mean ± SEM for 5 reps)

Isolate code and species	Place of origin	Degree of rotting (arc-sine transformed % of surface browning) at 7 days
7 *C. musae*	Windward Islands	90 ± 0.00^{a}
73 *Colletotrichum* sp.	Jamaica	90 ± 0.00^{a}
114 *Fusarium* sp.	Jamaica	90 ± 0.00^{a}
102 *Fusarium* sp.	Jamaica	76.84 ± 8.30^{ab}
12 *Fusarium* sp.	Windward Islands	76.13 ± 6.35^{abc}
10 *Fusarium* sp.	Windward Islands	72.53 ± 9.05^{bc}
113 *Fusarium* sp.	Jamaica	68.31 ± 11.66^{bcd}
112 *Colletotrichum* sp.	Jamaica	57.68 ± 2.89^{cde}
86 *Colletotrichum* sp.	Jamaica	56.06 ± 4.87^{def}
47 *Colletotrichum* sp.	Costa Rica	49.67 ± 2.20^{ef}
120 *Fusarium* sp.	Jamaica	38.53 ± 5.46^{fg}
108 *Colletotrichum* sp.	Jamaica	29.22 ± 1.63^{gh}
Control		18.44 ± 0.00^{h}
SED		7.81

^{a,b,c,d,e,f,g,h} Values followed by the same letter are not significantly different at $P = 0.05$

Screening for fungicide sensitivity

Three out of the six *Colletotrichum* sp. strains tested had TBZ ED_{50} values <1µg / ml, one in the range of 1-10µg /ml and two in the range >10µg/ml. The highest value recorded was 75µg/ml. All seven of the *Fusarium* sp. isolates tested had ED_{50} values between 1 and 10µg / ml as did the *Botryodiplodia theobromae* isolate. An unidentified but frequently occurring pathogenic species also had an ED_{50} value of <1µg / ml.

Isolation of putative biocontrol agents

Of thirty-one putative biocontrol agents isolated, seven gave promising results when compared with the control given by the standard TBZ fungicide against the pathogenic *C.musae* strain. In addition to the fungi, seven bacterial isolates with biocontrol potential were obtained from the banana leaves sampled. The identification of these and the fungi, along with the preliminary screening of the bacterial isolates, is in progress.

DISCUSSION

Isolation of fungi from infected crowns

The species isolated in this study were, on the whole, the same as those found in previous surveys to be involved in the crown rot complex. These are *Colletotrichum musae*, *Fusarium* spp., *Botryodiplodia theobromae*, other *Colletotrichum* spp., *Nigrospora* spp, *Curvularia* spp. and *Verticillium theobromae* (Johanson and Blazquez, 1990; Griffee and Burden, 1976; Wallbridge, 1981). In this study however *Curvularia* spp. appeared to be present in higher numbers from Jamaican fruit than has previously been found.

As reported by previous researchers the two most common groups of fungi isolated from each of the locations were *Colletotrichum* spp. and *Fusarium* spp. However, several differences between the locations can also be seen. The number of isolations per hand differs considerably from place to place. Sample hands from Jamaica yielded, on average, 16 different isolates. Windward island hands gave a similar yield of 18 isolates per hand. However, hands from Costa Rica yielded much fewer isolates, averaging only 7.7 isolates per hand. This could be due to the fact that both Jamaica and the Windward Islands have a larger crown rot problem than Costa Rica and so the pathogens are possibly more prevalent in the post-harvest environment in these countries. Hands from the Dominican Republic yielded yet higher sample numbers with an average of 26 isolations being made per hand. Despite this higher sample rate the Dominican republic fruit gave the lowest range of different organisms with 87% of the total isolations being *Colletotrichum* spp. or *Fusarium* spp. alone. This marked difference in range of organisms is likely to be because the Dominican republic fruit had not been treated. As such none of the more aggressive primary pathogens will have been reduced by fungicides and so secondary invaders will not have had the opportunity to colonise the cut crown tissue to the extent seen in the other countries.

Pathogenicity testing of fungi isolated from banana crowns

The results (table 2) show the isolates tested differ in terms of pathogenicity, broadly falling into three main groups; highly pathogenic isolates, moderately pathogenic isolates and isolates showing low pathogenicity. Of the three isolates in the highly pathogenic group two are *C. musae* strains. The moderately pathogenic group consists solely of *Fusarium* sp. strains and those in the lower band are all, with the exception of isolate 120 (a *Fusarium* sp. strain), other *Colletotrichum* sp. strains. These findings are similar to those of both Krauss (1996) and Finlay (1991) who also found *C. musae* to be more pathogenic than *Fusaruim* spp. at similar inoculation levels. Isolate 108 was found not to differ significantly from the control at P=0.05 and is therefore considered to be a non-pathogenic strain offering potential for development as a competitive biocontrol agent. To confirm pathogenicity of an organism re-isolation tests and re-inoculation were carried out to test Koch's postulates fully. The reisolation of fungi was successful in most cases although those disks showing fewer symptoms, in particular the control replicates, yielded some bacteria and unknown fungi. This suggests that micro-organisms had become established fairly deep into the peel tissue prior to sterilisation and questions whether inoculation studies would not be better performed on younger fruit in which deep seated infections would have had less chance to establish.

Screening for fungicide sensitivity

The results obtained suggest that tolerance to TBZ is present among fungi isolated from both treated and non-treated fruit. Of a sample size of just six, two *C. musae* isolates (33%) had ED_{50} level >10μg/ml highlighting the growing need for alternative crown rot treatments. The fact that one of these isolates came from an untreated hand demonstrates that tolerance is prevalent among natural fungal populations irrespective of current treatment. Following these findings it is felt that the survey should be extended to include other fungicides used for crown rot control and a larger number of sample isolates.

Isolation of putative biocontrol agents

The results obtained thus far are encouraging with seven potential biocontrol agents identified in these preliminary studies. Further isolations from a wider range of banana material are needed to increase this inoculum base. Following preliminary screens all promising biocontrol isolates will be further investigated to look at host range, fungicide tolerance and specific modes of action. Factors such as efficiency, pathogenicity and dosage required will also be investigated in detail.

REFERENCES

Finlay A R (1991). Crown rot control in banana. *Natural Resources Institute extra-mural contract X0051*, report for the period June 1990- August 1991.

Griffee P J; Burden O J (1976). Fungi associated with crown rot of boxed bananas in the Windward Islands. *Phytopathologische Zeitschrift* **85**, 149-158.

Jeger M J; Jeffries P (1988). Alternatives to usage for disease management in the post harvest environment. *Aspects of Applied Biology* **17**, 47-55.

Johanson A; Blazquez B (1990). Fungi associated with banana crown rot on field-packed fruit from the Windward Islands and assessment of their sensitivity to the fungicides prochloraz, thiobendazole and imazalil. *Crop Protection* **11**, 79-83

Krauss U (1996). Establishment of a bioassay for testing control measures against crown rot of banana. *Crop Protection* **15**, 269-274.

Wallbridge A (1981). Fungi associated with crown rot disease of boxed bananas from the Windward Islands during a two year survey. *Transactions of the British Mycological Society* **77**, 567-577.

Wilson C L; Pusey P L (1985). Potential for biological control of post harvest plant diseases. *Plant Disease* **69**, 375-378.

Advancement of ideas for the use of *Pasteuria penetrans* for the biological control of root-knot nematodes (*Meloidogyne* spp.)

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ABSTRACT

Current environmental awareness has led to a greater demand for alternative (non-chemical) pest control strategies. In the late 1970s early 80s a succession of reports by several workers linked the natural suppression of root-knot nematodes to the presence of a bacterial parasite. It was noted that nematode control was similar to that usually obtained with nematicides. Fuelled by these encouraging reports, research into the potential of *Pasteuria penetrans* for the biological control of root-knot nematodes - *Meloidogyne* spp. became established. Since 1986 14 PhDs, a similar number of MSc projects and studies made by visiting research fellows have been undertaken at The University of Reading. This work highlights previously unpublished data and discusses how the understanding of this obligate parasite has developed. Aspects of its biological requirement, specificity, effects of crop-host and the epidemiology are reviewed.

INTRODUCTION

Pasteuria penetrans (Pp) is an obligate spore-forming parasite and one of the most efficient natural enemies of root-knot nematodes, *Meloidogyne* spp. (RKN) Stirling (1984). The non-motile spores fortuitously attach to infective second stage juveniles (J_2s) in the soil as they seek a host root. Juveniles may become encumbered with vast numbers of spores; it is generally accepted that juveniles with a burden greater than 15 are less likely to be able to invade a host. Spores germinate after the nematode has invaded the host and established a feeding site. If the *Pasteuria* infection is successful, no eggs will be produced and the female will be full of spores.

Most of the early work at Reading focused on attachment studies and proving that *Pasteuria* would control RKN in pot experiments using different Pp populations originating from several countries. Often experiments were conducted using blends of different Pp isolates, this counteracted specificity between RKN species and Pp populations but created problems in repeating experiments and in some cases made publication of results difficult. It also became apparent how critical temperature would be for experimental work and for the mass production of spores. Systems to produce *Pasteuria penetrans in vitro* have to date been unsuccessful (Bishop & Ellar, 1992). Therefore mass production *in vivo* has been a particular interest in recent years.

EFFECT OF HOST CROP AND NEMATODE SPECIES ON SPORE PRODUCTION

In the endeavour to improve *in vivo* production of *Pasteuria penetrans* spores, several projects have studied the effects of the plant host. All experiments have followed the production methods of Stirling & Wachtel (1980).

In a glasshouse/pot experiment Richter (1991) encumbered juveniles of *M. javanica* with 4-7 spores of a Pp blend and compared tomato, marrow, okra and cowpea at three different inoculation densities. In a similar pot experiment in Ecuador, Triviño (1996) using 3000 *M. incognita* J_2s per pot encumbered with 3-7 spores (Pp Ecuador) compared eight nematode-susceptible crops for their spore production capability (Table 1).

Table 1. Spore production x 10^4/mg on different host crops at different inoculation densities with nematode juveniles encumbered with 3-7 spores/juvenile.

	Nematode density			
	2000	3000	5000	10000
	M. javanica	*M. incognita*	*M. javanica*	*M. javanica*
Cultivars				
Tomato cv Walter		40.2	-	-
Tomato cv Tiny Tim	6.3	-	38.1	22.8
Mung Bean	-	34.0	-	-
Marrow	7.1	-	18.6	35.3
Okra	6.2	-	17.0	21.2
Cowpea	11.2	15.0	14.5	9.3

Richter (1991) showed marrow as producing the highest spores/mg dry powder (353,000 spores/mg), whereas Triviño (1996) rated tomato cv Walter (400,000 spores/mg) the best host, followed by mung bean. She also suggested that mung bean may be a useful crop for increasing Pp in the field as it needs little care or irrigation. In both these experiments cowpea gave poor results, only producing 100-150,000 spores/mg. Richter (1991) found that okra did not perform well, however Giannakou & Gowen (1996) favoured okra as a host plant for *Pasteuria* production. Giannakou & Gowen (1996) and Giannakou (1998) also compared production of endospores/female in relation to plant host and inoculation density.

Table 2 shows a reduction in spore production per female with an increase in nematode density, proving that there is a nematode density/host plant tolerance level. The hypothesis put forward by Gowen & Channer (1988), and tested again by Richter (1991), that pruning root systems increases available invasion sites and thus increases Pp production, may not increase the overall production of *Pasteuria* spores, though the total number of infected females may be greater. It is also interesting to note (Table 2) that the reduction in spore production per female at increased nematode densities (up to 70% with tomato) is greater than the reduction of eggs/egg mass in the control treatments (Table 3). This suggests that

Pasteuria infection may be a greater drain on the resources of the host plant than the nematode parasite alone.

Table 2. Numbers of *Pasteuria penetrans* endospores produced in *Meloidogyne javanica* females extracted from tomato and okra roots after inoculation at three population levels.

Plant host	Juvenile inoculum per plant 1000	3000	10000	Mean
Okra	660,200	540,730	299,370	500,100
Tomato	466,020	354,340	141,520	320,670
Mean	653,691	447,540	220,450	

Treatment effects: Host, $P < 0.001$, SED = 38.04: Inoculation level, $P < 0.001$, SED = 46.59; Interaction, $P > 0.05$.

Table 3. Numbers of eggs produced from *Meloidogyne javanica* females reared in tomato and okra roots after inoculation at three population levels.

Plant host	Juvenile inoculum per plant 1000	3000	10000	Mean
Okra	730	742	579	683
Tomato	528	533	447	502
Mean	629	637.5	513	

Treatment effects: Host, $P < 0.05$, SED = 50.95; Inoculation level, $P > 0.05$; Interaction, $P > 0.05$.

Given optimum conditions for the *Pasteuria*, the more susceptible the host plant to RKN the better it will be for spore production. Zibanda (1996 - unpublished) using tomato showed that different cultivars of the same host crop gave differing levels of spore production.

Production may depend on the RKN species. De Costa (1991) reported that *M. javanica* females produced greater numbers of spores compared to *M. incognita* females and that this was probably related to the size of female. Giannakou (1998) also found that the size of

female was relevant to spore production per female, and that size of female may again be influenced by the host crop.

SPECIFICITY AND SPORE ATTACHMENT

Pasteuria populations are highly specific to RKN species and populations. Stirling (1991) suggests that much of the published data should be treated with caution as much of it is based on spore attachment to the nematode and not infection of the female by the bacterial parasite. Each time a *Pasteuria* isolate is passaged its genomic make-up could change making comparisons between either attachment or pathogenicity tests difficult. No-one has yet reported (or succeeded in producing) single spore lines.

Inconsistent methodology makes comparisons between attachment data impossible. Many factors affect results obtained during attachment studies - nematode density; spore density; maturity and age of spores; age of juveniles; temperature; time of exposure and pH. Stubbs (1998) reported that levels of attachment might vary even when using dishes of different size.

Following an observation that after a period of time spore attachment per juvenile did not increase, Weekes (1988) found that by continuously changing the J_2s within a spore suspension each 24 h that with each cohort the level of spores/J_2 soon declined. This suggests that only a proportion of the spores in a suspension is capable of attaching to that particular population of RKN. This finding appeared to be of epidemiological importance. This begged the question, were the remaining spores non-viable or were they not capable of attaching to that particular nematode species/population?

Later, Ladu (1996) re-addressed this question and probed suspensions of spores with different species of RKN in different sequences. In summary, her results confirmed those of Weekes (1988) that only less than 5% of spores in a suspension will attach to nematodes. With Pp populations originating from S Africa (Pp3) and Ecuador greater numbers of spores attached to *M. incognita* (Malawi) than to *M. javanica* (Malawi) or *M. arenaria* (country of origin unknown). Ladu's results showed that if *M. incognita* was first in the sequence greatest numbers attached to these nematodes and few if any attached to *M. arenaria* and *M. javanica* when they were used 2nd or 3rd. Interestingly, if the attachment sequences started with *M. arenaria* the spores of Pp3 attached in relatively high numbers as they did to *M. javanica* and *M. incognita* when probed 2nd and 3rd respectively. This indicated that in the Pp3 population there were some spores that could attach to more than one species of nematode. Studies on this theme although difficult to carry out will continue.

THE EFFECT OF TEMPERATURE ON *PASTEURIA* PRODUCTION

Successful production of Pp is highly dependent on temperature. Stirling (1981) advocated the use of degree days to predict maturity of *Pasteuria*. Giannakou (1988) presented data supporting the hypothesis that the development of Pp is faster when the heat units are accumulated at temperatures close to the optimum.

Samaliev (1996 - unpublished) compared spore production of two Pp population, Pp3 and from the Ivory Coast, at 20, 25 and 30°C. Using a modified sealed container technique, 1000 J_2 encumbered with 1-10 spores were inoculated on potato roots cv. Cara. Roots were harvested after 700-800 degree days had accumulated at the respective temperatures.

Table 4. Spore production x 10^6 per root system of potato cv. Cara inoculated 1000 J_2 encumbered 1-10 spore juvenile.

	Temperature		
	20°C	25°C	30°C
Pp Ivory Coast	14[a]	85[a]	89[a]
Pp3	11[a]	13[b]	72[a]

Means followed by the same letter are not significant < 0.05

Table 4 shows that the Pp population from Ivory Coast has a wider temperature range than Pp3. This has implications for field deployment; some *Pasteuria* populations may be better suited to particular climatic regions.

CONCLUSIONS

Successful use of *Pasteuria penetrans* within an IPM project will require further on-farm evaluation. Alongside this will be a need to develop a reliable system for the mass production of *Pasteuria* spores. Inducing root-knot suppression within a system of continuous root-knot susceptible crops is not without risks. The introduction of root-knot nematode non host/poor host crops on the epidemiology of the bacteria needs to be assessed. Further studies on the viability and specificity of spores within *Pasteuria* populations are needed to understand relationships between spore density and nematode management.

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REFERENCES

Bishop A; Ellar D J (1992). Attempts to culture *Pasteuria penetrans* in *vitro*. *Biocontrol Science and Technology* **1**, 101-114.

De Costa D (1991). Efficiency of *Pasteuria penetrans* in controlling *M. javanica* in different host plants and some factors affecting *in vivo* production. MSc Thesis, Department of Agriculture, The University of Reading.

Giannakou I (1998). Aspects on the use of *Pasteuria penetrans* infecting root-knot nematodes (*Meloidogyne* spp.). PhD Thesis, Department of Agriculture, The University of Reading.

Giannakou I O; Gowen S R (1996). The deployment of *Pasteuria penetrans* as affected by different plant hosts. *Brighton Crop Protection Conference, Pests and Diseases* **1**. 393-398.

Gowen S R; Channer A G (1988). The production of *Pasteuria penetrans* for control of root knot nematodes. *Brighton Crop Protection Conference, Pests and Diseases* (3), 1215-1220.

Ladu R D M (1996). Capability of two populations of *Pasteuria penetrans* to attach to different species of *Meloidogyne* spp. MSc Thesis, Department of Agriculture, The University of Reading.

Richter M (1991). Evaluation of different host plant species and potting media for the *in vivo* production of *Pasteuria penetrans*. MSc Thesis, Department of Agriculture, The University of Reading.

Stirling G R (1981). Effect of temperature on infection of *Meloidogyne javanica* by *Bacillus penetrans*. *Nematologica* **27**, 458-462.

Stirling G R (1984). Biological control of *M. javanica* with *Bacillus penetrans*. *Phytopathology* **74** (1), 55-60.

Stirling G R (1991). *Biological Control of Plant-Parasitic Nematodes: Problems and Prospects.* Wallingford: CABI.

Stirling G R; Wachtel M F (1980). Mass production of *Bacillus penetrans* for the biological control of root-knot nematodes. *Nematologica* **26**, 308-312.

Stubbs V (1998). The use of *Tagetes erecta* and *Pasteuria penetrans* within crop rotations for the control of *Meloidogyne* spp. in microplots in Zimbabwe. PhD Thesis, Department of Agriculture, The University of Reading.

Triviño C G (1996). The occurrence of *Pasteuria penetrans* infecting root-knot nematodes (*Meloidogyne* spp.) in vegetable field in Ecuador and its potential role in nematode management. PhD Thesis, Department of Agriculture, The University of Reading.

Weekes C S A (1988). Studies on *Pasteuria penetrans* and its production on hosts of *Meloidogyne incognita*. MSc Thesis. Department of Agriculture, The University of Reading.

The effects of an organosilicone/latex-based adjuvant and the fungus *Trichoderma* on the efficacy of copper sprays used for the control of witches'broom disease in cocoa

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ABSTRACT

During 1998 field trials were undertaken to assess the effects of an adjuvant and a novel strain of the biocontrol fungus *Trichoderma* on the efficacy of cuprous oxide sprays used for the control of witches' broom disease in cocoa. The adjuvant used was a novel organosilicone/latex-based mixture (Tactic). Treatments comprised cuprous oxide, Tactic, cuprous oxide plus Tactic, cuprous oxide plus Tactic and *Trichoderma*, and an untreated control. Each treatment was replicated four times using plots with ca. 50 mature cocoa trees. All treatments were applied monthly from March to September using a backpack mistblower. Throughout the treatment period, and for two months afterwards, assessments of healthy and infected pods, green and dry axillary and terminal vegetative brooms, and vegetative cushion brooms were made. Preliminary results show that all of the copper-based treatments may be effective at preventing disease development. However, which of these treatments is most effective, and whether the fungus or the adjuvant are able to enhance disease control, was not yet clear following the first five months of the research.

INTRODUCTION

Witches' broom, *Crinipellis perniciosa*, is the single most important factor constraining cocoa production in the main cocoa growing region of Brazil (Bahia state) (Aitken, 1997). Since 1989, when the disease was first detected in Bahia, yields have consistently declined (Periera *et al.*, 1990). In 1997 the total cocoa bean harvest in Bahia was ca. 40% of its level in 1989. Current strategies for the control of the disease in Bahia include a mixture of phytosanitation and fungicide application (Evans, 1981; Rudgard *et al.*, 1993). In Bahia, the recommended fungicide is cuprous oxide, which should be applied monthly for 5 to 6 months from March. Guidelines for phytosanitation comprise pruning of diseased tissue in March/April and October/November (Aitken, 1997). Despite these recommendations, yields have continued to decline.

Current research attempts to improve the control of witches' broom disease include trials with other fungicides, trials with biological control agents, and breeding attempts to identify clones with resistance to the disease (Laker & Rudgard, 1989; Rudgard *et al.*, 1993; Purdy & Schmidt, 1996; Bastos, 1996). In this paper we report the results of a field trial undertaken in 1998 that was designed to investigate whether improvements in disease control could be realised by (1) enhancing the efficacy of cuprous oxide sprays by adding an adjuvant to the spray tank and (2) by combining chemical and biological control agents. The adjuvant assayed was a novel spreader/sticker and the biological control agent assayed was a novel strain of *Trichoderma* (strain TVC) isolated from the Amazon region (Belém) of Brazil (Bastos, 1996).

MATERIALS AND METHODS

Experimental Design

The field trials were carried out on an area at the São Jorge farm, near Itabuna, Bahia, Brazil. Plots were marked out that comprised ca. 50 mature cocoa trees each (Figure 1). All treatments were replicated four times and all assessments were made from five randomly picked trees located in the center of each plot. Treatments were allocated randomly to the plots shown in Figure 1.

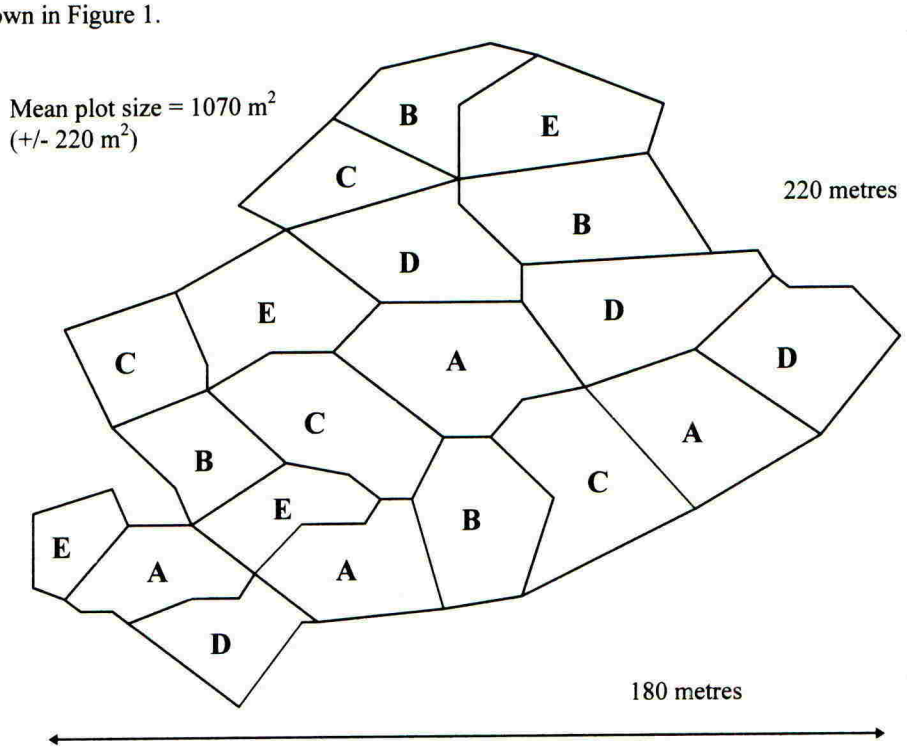

Figure 1. Experimental layout at São Jorge farm. Key for treatments: A = control, B = cuprous oxide, C = tactic, D = cuprous oxide + tactic, E = cuprous oxide + tactic + *Trichoderma*.

Treatments

All treatments were applied with a motorised backpack sprayer at a volume application rate of 400 ml per tree. Spraying was undertaken monthly from April through to August (see below). The concentrations of cuprous oxide (Copper Sandoz) and Tactic were 3% and 0.75%, respectively. Both were diluted with tap water. *Trichoderma* cultures were produced on-site using a simple 20 L fermentor with molasses yeast extract as the substrate (Lewis & Papavizas, 1983). These cultures were composed of chlamydospores (10^7/ml) and conidia (10^8/ml).

Disease Assessments

In each plot 5 centrally located trees were randomly selected on each sampling occasion for disease assessment. Assessments were made of (1) the number of healthy pods, (2) the number of pods infected with *Crinipellis perniciosa*, (3) the number of pods infected with *Phytophthora palmivora*, (4) the number of green axillary and terminal vegetative brooms, (5) the number of necrotic axillary and terminal brooms, and (6) the number of vegetative cushion brooms. Only pods over 6 cm in length were assessed. In addition to the above, at each harvest, assessments of pods were made by measurements 1 - 3 above.

Assessment and Spraying Schedule

The spraying and assessment schedule for the field trial is given in Table 1. The trials ran from March until November 1998 and comprised a cycle of disease assessment, chemical application, and harvest. Each of these operations took a week to complete for all of the replicate plots. Pods are only susceptible to disease in the first twelve weeks of their development, which takes 5 - 6 months from flower pollination to pod maturity (Fulton, 1989; Rudgard *et al.*, 1993). In Bahia, it is the pods that are harvested in September/October that usually suffer the most from witches' broom disease.

Table 1. Disease Assessment and Spraying Cycle.

Disease Assessment	Spray Application	Harvest
30th March	6th April	20th April
4th May	11th May	25th May
8th June	15th June	29th June
13th July	29th July	3rd August
17th August	24th August	8th September
21st September	n/a	13th October
26th October	n/a	16th November
30th November	n/a	n/a

Data Analyses and Presentation

The data from the trials were analysed, and are presented, as the mean assessment parameter (+/- 95% confidence limits) recorded for each treatment through time. In this paper we present

preliminary results for the number of vegetative brooms and for the number of infected pods. In addition, we also present data for the percentage disease incidence recorded for each treatment calculated from the relationship between the number of diseased and healthy pods assessed on each sampling occasion. A more complete analysis of the data will be given at a later date following completion of the trial.

RESULTS

Figure 2 shows the mean numbers of axillary and terminal vegetative brooms recorded per tree in each of the treatments over the first five assessment dates. These data were therefore collected following four spray application rounds. The data, although not statistically significant, show one clear trend in the consistent increase in the number of brooms recorded in control plots. The number of brooms recorded in all other treatments remained approximately constant at ca. 1 - 2 brooms per tree following a decline from pre-treatment application numbers of ca. 2 - 4 brooms per tree. No differences between the other treatments were therefore apparent at this stage. However, all of these treatments did appear to be having some effect upon disease development.

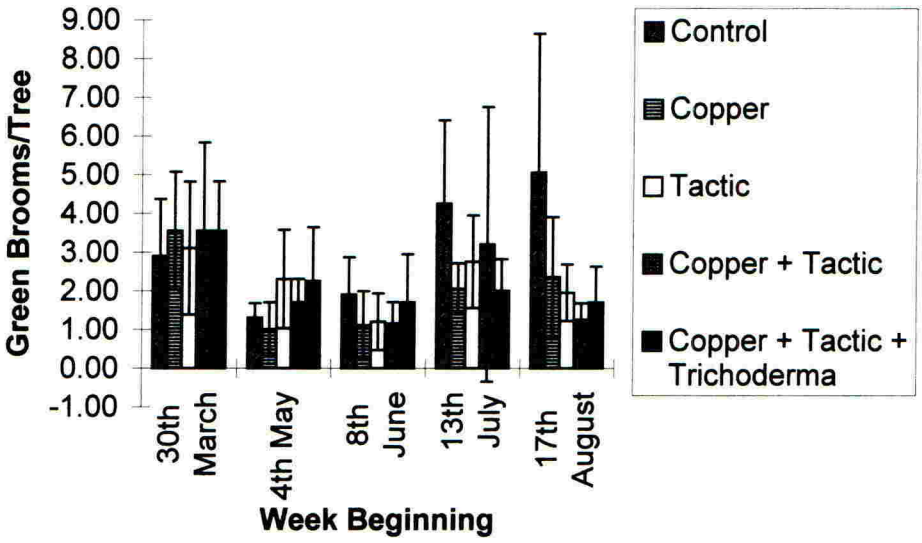

Figure 2. The mean number of green brooms (+/- 95% confidence limits) recorded in treatment plots over the first five sampling occasions.

Figure 3 shows the mean number of infected pods per tree in each treatment recorded over the first five sampling occasions. The data show that by the fifth sampling occasion statistical differences existed between treatments. Numbers of infected pods were highest in the control and adjuvant plots while numbers were lowest in all the copper-based treatments. No statistically significant differences were recorded between the copper-based treatments.

Figure 4 shows the percentage disease incidence recorded per tree over the first five sampling occasions. Disease incidence over these sampling occasions, over all treatments, was low and varied between ca. 1 - 9%. However, by the fifth assessment occasion the data suggest that the most effective treatments were copper-based.

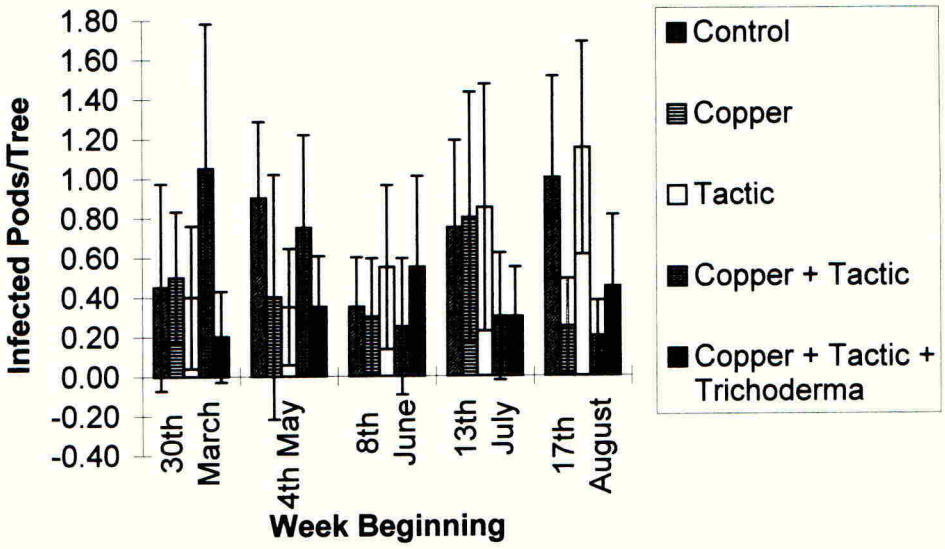

Figure 3. The mean number of infected pods (+/- 95% confidence limits) recorded in treatment plots over the first five sampling occasions.

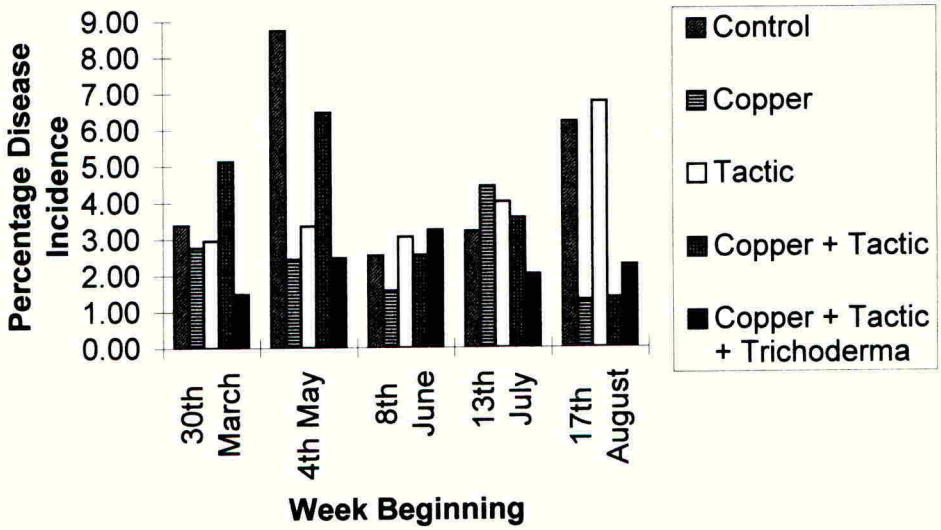

Figure 4. The percentage disease incidence recorded in each treatment over the first five sampling occasions.

DISCUSSION

The data presented in this paper are preliminary. Statistical differences in treatments were recorded by the fifth assessment period with the data indicating that all of the copper-based treatments may be effective in slowing disease development although statistical differences between these treatments were not yet evident. Overall disease incidence has so far been low.

These results are to be expected. In the case of the low disease incidence over the period March - July, the evidence from Bahia suggests that the early crop typically usually escapes infection (Aitken , 1997). It is the main, late crop, in September - October that usually suffers most from the disease. Data, yet to be collected, will indicate whether this crop has been protected from the disease. The lack of any statistical difference between the copper-based treatments is explained by the fact that cuprous oxide works as a protectant, mainly during the first 12 weeks of pod development when pods are most susceptible. There will therefore be an interval of 5 to 6 months to allow for pod development before which differences in treatment effects may be seen. Once again, we would not expect to realise any differences between treatments until much later in the season. Further, and more detailed analyses, will be undertaken once the trial is completed in late November.

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REFERENCES

Aitken, W M (1997). Chemical and phytosanitationcontrol of Witches' Broom for cocoa. In: *Proceedings of ACRI/CAOBISCO/IOCC Witches' Broom Workshop.*

Bastos, C N (1996). Potencial de Trichoderma viride no controle da vassoura-de-bruxa (*Crinipellis perniciosa*) do cacaueiro. *Fitopatologie Brasil* **21,** 509-512.

Evans, H C (1981). Witches' Broom Disease- A Case Study. *Cocoa Growers' Bulletin* **32,** 5-19.

Fulton, R H (1989). The Cacao Disease Trilogy: Black Pod, Monilia Pod Rot, and Witches' Broom. *Plant Disease* **73,** 601-603.

Laker, H; Rudgard, S A (1989). A Review of the Research on Chemical Control of Witches' Broom Disease of Cocoa. *Cocoa Growers' Bulletin* **42,** 12-24.

Lewis, J A; Papavizas, G C (1983). Production of chlamydospores and conidia by Trichoderma spp. in liquid and solid growth media. *Soil Biology Biochemistry* **15,** 351-357.

Periera, J L; Ram A; Figueiredo J M De; Almeida L C C De (1990). First occurrence of Witches' Broom disease in the principal cocoa growing region of Brazil. *Tropical Agriculture* **67,** 188-189.

Purdy, L H; Schmidt, R A (1996). Status of Cacao Witches' Broom: Biology, Epidemiology and Management. *Annual Review of Phytopathology* **34,** 573-594.

Rudgard S A; Maddison A C; Andebrhan T (1993). *Disease Management in Cocoa.* Chapman & Hall, New York.

Farmer participatory research in spraying machinery development in Colombian coffee

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ABSTRACT

A two-year research and development programme resulted a in system of low volume application of fungicides to coffee trees which, in season long trials, gave control results at least as good as conventional systems of application. In order to develop the system to field use, a farmer participatory research phase was undertaken with the aim of identifying engineering and other potential constraints to adoption. This phase highlighted a number of engineering and ergonomic problems that were readily addressed. In terms of operator perceptions, the major difficulty was identified as a lack of sufficient deposit at the base of the trees grown on steep slopes. Although there will be a limit on the conditions under which the low volume system will be effective, in most cases the failure to see the spray by the operators is the main cause of this perception. The farmer participatory research phase has confirmed impressions from the development phase, as well as highlighting areas requiring further attention, particularly regarding the methods of transferring this technology to farm use.

INTRODUCTION

Coffee leaf rust (*Hemileia vastatrix*) reached the Colombian coffee growing zone in the early 1980s (Waller *et al.*, 1994). As the first major crop protection problem in Colombian coffee, initial systems of control were based on Kenyan and Brazilian experience, modified in the light of local circumstances. These initial systems of control were based on the use of copper oxychloride as a protectant fungicide applied between 4 and 6 times per season. Application was at high volumes (250–500 litres/ha) using a variety of equipment, including pressure retaining sprayers, semi-stationary pumps or conventional knapsack sprayers. However, it was soon recognised by the Colombian Coffee Federation that the particular agronomic and topographical circumstances of coffee cultivation in Colombia made the use of high volumes especially difficult. The nature of the terrain on which coffee is grown, usually on steep slopes, and the planting density of the trees (up to 10,000 trees per hectare planted at a 1 m by 1 m spacing) makes conventional application systems and volumes slow and difficult, and as a result expensive.

A research programme was initiated to examine alternative systems of control, based on the use of low volume application rates (less than 50 litres/ha). As a result of a two-year research and development phase, which was founded on previous work on non-conventional application systems - particularly electrostatic sprayers (Aston, 1991), an experimental machine was developed which was able to control coffee leaf rust to a level as good as conventional high

volume systems in year long field trials, but using a much reduced application volume. This machine and system of use, which became known as the Motax, was shown to be biologically as efficient as conventional systems, but with the additional advantage of much reduced time of application.

The crop protection situation in Colombian coffee was further complicated by the arrival of the coffee berry borer (*Hypothenemus hampei*) in 1990, which has subsequently spread to over half of the 1 million hectares of coffee in the country. Control of this insect is based on an integrated strategy, including the application of the insect pathogenic fungus *Beauveria bassiana*. Initial trials with the machine for the application of this fungus has proved encouraging (Florez *et al.*, 1997).

The two-year research phase of low volume spraying led to a prototype machine, based on deposit parameters required to achieve control. However, all of the research undertaken had been conducted within the confines of a research institution. In order to successfully introduce the system of low volume application to farmers, a development phase was undertaken to improve the machine from the point of view of field practice. This phase, the farmer participatory research (FPR) phase, was designed to examine, under practical field conditions, the limitations to potential adoption of the low volume technology based on user response. As such the aim was to identify practical modifications to the design of the machine and the system of use which would make the technology more user friendly.

Development

The two-year research phase was focused on defining, under local conditions, the required biological and physical deposition characteristics for successful control of leaf rust. This phase involved physical, biological and economic evaluations of potential control systems, and involved both laboratory and field work. Laboratory studies of excised coffee leaves sprayed with defined doses of copper oxychloride and inoculated with coffee leaf rust spores showed that sufficient inhibition of germination of the spores could be achieved if 30 drops per cm^2 of a 3% copper oxychoride suspension in 100 µm drops was deposited (R Aston, in prep.). Concurrently, field trials designed to measure the deposit obtained under various systems of droplet production, system of drop movement and agronomic conditions were undertaken. The machine used for this was a experimental machine in which factors such as drop size, air velocity and flowrate (and thus volume application rate) could be changed. Once the means of achieving the required deposit parameters were defined, a prototype machine with these factors fixed was designed and built. This machine was subject to laboratory and field evaluation by research staff, and found to meet the deposit criteria required for control. Engineering and ergonomic improvements were identified, and were incorporated and tested. A pre-production model was designed from this, and a number of these machines were constructed for the FPR.

Description of the prototype machine

The machine used for the FPR was based on the principles developed during the research phase. Droplets are produced by a spinning disc, mounted in front of an axial fan which is driven by a 2-stroke engine of 33 cc capacity, rotating the fan at 6,500 rpm. The disc is driven by a propeller which is centrally mounted to the airstream produced from the fan. Droplet size on the FPR machines was fixed at 100 µm VMD. Flowrate is altered by means of restrictors,

which can be changed to alter the volume applied. The 7 litre tank is mounted high on the machine, in order to gravity feed the disc, and to improve balance. The machine is back mounted, and the droplets move away from the operator with the airstream. In order to ensure coverage of two rows of coffee in a single pass, the machine is fitted with a handle that allows the operator to move the motor and disc assembly from side to side. The handle can be used either on the right or left of the machine. Further, the fan/disc assembly can be moved in the vertical plain (i.e. up or down) through three positions to take into account the height of the tree. The controls (accelerator and liquid on/off switch) are located low on the frame of the backpack. The total weight of the machine is 20 kg when ready for spraying.

Farmer participatory research

Thirteen pre-production machines were constructed. Farms for inclusion in the FPR were selected throughout the coffee zone based on a number of factors, including farm size, technology of production, topography and planting distance of those thought likely to adopt low volume technology. This was done in conjunction with the local extension service, who were closely involved with the FPR process.

Farmer owners and operators were given full explanations on the nature of the machine and trained in its use. Farmers were encouraged to use the machine as part of their normal crop protection programme. This included the application of copper for control of leaf rust, as well as *B. bassiana* for the control of berry borer. Technical support was given to the farmers as required, as part of a normal programme of visits by extension and research staff.

The farmers were required to keep a record of the hours used for each machine, together with their perceptions of the machine and its use under field conditions. No data were requested on the biological effectiveness of the applications, as the aim of the FPR was to examine engineering and ergonomic questions related to farmer use, together with perceptions which would hinder adoption on a wide scale.

At the end of the ten month period of the trial, farmers and operators were questioned on their experience of the machine. In addition to the formal questionnaires, general discussions were held to understand the background to the answers. Extension staff were also involved in the evaluation exercise.

RESULTS

The machines were used on average for 127 hours (for a total operating time of 1,657 hours), although there were wide variations between farms. The lowest use was 40 hours, and the highest 280 hours (Table 1).

Spray operators were generally very experienced, with over 75% having more than three years spraying experience. However, the experience with motorised back mounted equipment was comparatively low, with only 31% having used motorised equipment previously (all had used lever operated knapsack machines, and over 75% had used the pre-pressurised compression sprayers).

Table 1. Details of farms used in the FPR and machine use.

Farm number	Planting density (trees/ha)	Area of coffee (ha)	Machine use (hours)
1	6,900	160	75
2	8,000	70	60
3	6,900	25	280
4	10,000	100	80
5	5,000	160	160
6	9,500	40	40
7	5,000	150	115
8	4,700	80	40
9	6,666	24	262
10	6,900	80	207
11	5,900	42	145
12	3,703	70	120
13	5,000	60	73

Following the training, no operator reported difficulties in preparing the machine for use, or indeed in operating the machine. No operator found that the machine was too heavy, which was a concern during the research and development phase. Most conventional equipment is similar in weight to the 'new' machine, while other motorised machines can be significantly heavier when full. Time to empty the machine of liquid is approximately 30 minutes under the conditions used throughout the FPR; the pre-pressurised sprayer is emptied in about 50 minutes. On the overall design of the machine related to comfort of the operator, slightly more than half those involved in the FPR found that the straps were uncomfortable. During the research phase and in trials with the prototype equipment, it had been found that maximum comfort was obtained when the machine was mounted as high as possible; if it was mounted too low, then the weight of the machine tended to pull the operator off balance; at the same time this caused the discomfort on the hips of the operator. It is likely that this was the primary cause of the results found in the FPR. Over 75% of the operators reported that the machine produced significant vibration, almost equally split between vibration felt in the shoulders, on the back and through the handle. A further problem was identified as the noise produced by the machine. Measurements by Villalba (personal communication) had shown that the levels of noise produced were between 96 and 104 decibels (which was above the permitted levels of 90 Db without ear protection); in the absence of ear protection, 85% of the operators reported that the noise produced during application was high. However, over 50% of the operators considered that the use of ear protectors caused other problems – particularly due to the heat. A number of problems were reported with the construction of the machine. Some were caused by the vibration – for example loosening of screws. Other perceived problems were inherent in the design – a large number of operators reported that the tank was too high. However, it is not clear why they considered this was so, unless it was related to comfort as discussed above. Other problems reported included minor faults (although important in terms of field use), such as breakage of the feed tubes, caused by catching in branches of trees, difficulty in cleaning due to positioning of the fan guard, and other reasonably easily rectifiable faults. A further problem reported was that more than half of the respondents suggested that the on/off switch was

difficult to locate (it was positioned on the frame of the machine, so that the operator was required to feel for it).

Operationally, the machine presented mixed results in the opinion of the operators. The majority (85%) considered that the application to trees on flat plantations was good. This was largely attributed to good coverage of the plant. However, on slopes, a similar majority considered that the application was poor, largely due to the perception that the cover was poor. All of the operators reported that they adjusted the vertical angle of the fan to take into account the nature of the plantation. None of the operators found it difficult to walk with the machine in flat plantations, although two thirds reported that application on slopes was difficult. This reflects the fact that application using the machine is a continuous process, as opposed to the tree by tree application of conventional systems. This means that it is less easy for operators to rest, or to hold on to trees as they spray.

In terms of operator safety, the results were largely positive. The design of the machine is such that the droplets are blown away from the operator to the rear. Over 90% of the operators said that they felt no spray when the machine was used on flat coffee plantations, and nearly 85% reported no contamination when spraying coffee on slopes. The contamination that did arise always occurred at the end of the row, when the operator re-entered the block after completing the previous row. Some contamination of the operators arose from leakage from the tank neck; in these prototypes, the tank neck was fitted to the tank by means of bolts. Vibration is likely to have loosened these bolts. An operational problem with the design of the machine, with the spray being emitted behind the operator, is that it is difficult for a single operator to see when the machine is not spraying – for example through blockage or when the tank is empty. The operators themselves suggested a number of solutions to this problem, the most ingenious being the use of a small mirror to see behind them. Also it seemed that a number of operators were able to glance behind and check on the functioning.

Discussions were held with the extension workers, who have considerable experience of pesticide application to coffee. They identified a number of advantages of the machine for application, including increased workrate, better penetration and cover of the trees, and interestingly, the potential to reduce doses. They also highlighted reduced operator contamination. At the same time, they expressed some reservations on the general applicability of the machine to coffee cultivation in Colombia. In particular, as with the operators, they were concerned about cover on trees on slopes, and how as the inter-row distance increased, cover reduced. They also suggested that, particularly on tall trees, cover at the very top and very bottom of the tree could be poor.

CONCLUSIONS

Although the sample size is comparatively small, the FPR exercise generated interesting data, both confirming previous ideas and suggesting new ones. The results can be divided into two categories. Firstly, there are those data which feed directly into improved design. Secondly, there are results which are important because they highlight perceptions of farmers and operators which may not be technically valid, but which need to be addressed before the technology is adopted on a wide scale. In terms of engineering limitations of the machine highlighted by this study, most are relatively easily corrected. For example, excessive vibration

(both a problem to operators and as an underlying cause of other problems) has been addressed through the use of vibration damping mounting rubbers. The reduction of noise, through the use of improved exhaust design is being examined.

Other problems are largely ones of perceptions, the most important being the perception that the cover achieved by the machine when used on steep slopes is poor. Numerous studies on the deposition achieved by the machine under varying conditions of slope have shown that the deposit achieved is satisfactory (R Aston, unpublished data). However, as with most low and ultra low volume systems of application, the difficulty of seeing the deposit on the target can lead to erroneous conclusions by operators (Aston, 1997). At the same time, it is recognised that there will be limit (in terms of steepness of slope, height of coffee trees and row distance) at which the system of low volume application with the machine will not give acceptable control with the system of application recommended.

Many of the results from the FPR have been incorporated into developing the machine into a system capable of commercial exploitation. The commercially available machine is described by Povey *et al.* (1996). The FPR has, therefore, led to direct outputs which can be used in a practical way to improve the potential for adoption of the system of low volume application in Colombian coffee – and by extension other crops on which this system of low volume air assisted spraying may have a role.

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REFERENCES

Aston R (1997). The introduction of low volume CDA spraying for the control of invasive weeds in Colombian pastures. BCPC Conference – *Weeds, 1997,* **1,** 221-226.

Aston R; Sharp D G; Clayton J S (1991). The use of air assisted hand held ULV spraying for control of coffee leaf rust in Colombia. BCPC Monograph No **46** *Air Assisted Spraying in Crop Protectio,* pp. 151-160.

Florez E; Bustillo A; Montoya E, (1997). Evaluacion de equipos de aspersion para el control de *Hypothenemus hampei* con el hongo *Beauveria bassiana. Cenicafe (Colombia)* **48,** 92-98.

Povey G S; Clayton J S; Bals T E; (1996) A portable motorised axial fan air-assisted CDA sprayer : a new approach to insect and disease control in coffee. BCPC Conference - *Pests and Diseases, 1996,* **1,** 367-372.

Waller J M; Leguizamon J; Gill L F; Aston R; Cookman G P; Sharp D S; Ford M G; Salt D W (1994) Laboratory and Field Development of a CDA spraying system for control of coffee leaf rust (*Hemileia vastatrix*): an overview. BCPC Monograph No **59** *Comparing glasshouse and field pesticide performance,* pp. 261-266.