DECREASED BINDING OF CARBENDAZIM TO CELLULAR PROTEIN FROM *VENTURIA NASHICOLA* AND ITS INVOLVEMENT IN BENZIMIDAZOLE RESISTANCE

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ABSTRACT

The ^{14}C-carbendazim binding activity of cell-free mycelial extracts of benzimidazole resistant and sensitive isolates of *Venturia nashicola* has been determined. Extracts of the resistant isolates exhibited lower binding activity than that of the sensitive one, indicating that benzimidazole resistance might be based on a lower affinity of tubulin-like protein for benzimidazoles. However, no difference was observed in the binding activity between extracts of isolates with high, intermediate or weak resistance.

INTRODUCTION

During the last decade, benzimidazole-resistant strains of *Venturia nashicola*, the causal organism of scab of Japanese pear (*Pyrus serotina* var. *culta*), have frequently been detected from pear orchards in Japan where these fungicides failed to control the disease. Although the use of benzimidazole fungicides has been stopped or restricted in the orchards concerned, benzimidazole-resistant strains are still widely distributed throughout the country (Ishii *et al.* 1985).

In most cases, field populations of *V. nashicola* comprise a mixture of strains which differ in the level of resistance to benzimidazoles. Three levels of resistance, viz. high resistance, intermediate resistance and weak resistance, have been characterized and genetical data indicate that these are conferred by allelic mutations in a single gene (Ishii *et al.* 1984).

The primary mode of action of carbendazim is generally considered to be a binding to β-tubulin resulting in an interference with microtubule assembly which is essential for a

functional cytoskeleton (Corbet et al. 1984). The biochemistry and the genetics of benzimidazole resistance have been intensively studied in Aspergillus nidulans. In this fungus, the locus benA in which mutants confer either resistance or super-sensitivity to benzimidazoles was identified as the structural gene for β-tubulin (Sheir-Neiss et al. 1978). Binding studies have shown that resistance and super-sensitivity to carbendazim were based on changes in the binding activity of tubulin to this fungicide (Davidse 1975). The benA gene of A. nidulans has been sequenced and amino acid sequences important in the interactions between β-tubulin and benzimidazoles have been discussed (Morris et al. 1985). The involvement of mutation in β-tubulin gene with benzimidazole resistance has been suggested also in Saccharomyces cerevisiae (Neff et al. 1983) and Schizosaccharomyces pombe (Hiraoka et al. 1984).

Studies with plant pathogenic fungi have been limited but a correlation between affinity of tubulin for carbendazim and degree of sensitivity to benzimidazoles has also been observed in Penicillium expansum (Davidse 1982). Extracts of the benomyl-resistant isolate of V. inaequalis also had different binding properties from those of sensitive isolate. Binding of carbendazim to a tubulin fraction appeared to be lower but binding to other components of the extracts increased (Gasztonyi personal communication).

To elucidate the biochemical mechanism of benzimidazole resistance in V. nashicola in relation to its genetic basis, the binding activity of the cellular protein to the benzimidazole fungicide carbendazim was determined for the various isolates. The Plant Protection Service at Wageningen gave permission to use isolates of V. nashicola in the Netherlands where most of these studies were carried out.

MATERIALS AND METHODS

Isolates and culture conditions

Four monoconidial isolates of V. nashicola were used. The response of each isolate to carbendazim was classified as follows for MIC, minimal inhibitory concentration that gives 100% inhibition of mycelial growth: JS-18, sensitive (≤1 μg/ml); JS-27, weakly resistant (≤10 μg/ml>1 μg/ml); JS-40, intermediately resistant, (≤100 μg/ml>10 μg/ml); JS-137, highly resistant (>100 μg/ml). The isolates were obtained from scab lesions in pear orchards and kept on potato dextrose agar (PDA) slants at 10°C until use.

Mycelial discs of each isolate, cut from cultures grown on PDA at 20°C for 45 days were transferred to a liquid medium containing 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 1% glucose(wt/vol). After incubation at 20°C for one month, mycelia, collected from the cultures, were washed with sterile distilled water and homogenized aseptically. The

mycelial fragments thus obtained were used to inoculate the liquid medium described above and incubated at 20°C.

Preparation of mycelial extracts

Fresh mycelia, harvested from cultures in a liquid medium, were washed in cold 0.05M potassium phosphate buffer, pH 6.8 containing 0.1M KCl and 0.005M $MgCl_2$ (PKM_g solution, Davidse and Flach, 1977) and placed in a previously cooled (-20°C) X-Press Cell Disintegrator (LKB-Biotec) with 1 ml of PKM_g solution per gram wet weight of mycelium. After freezing, the mycelia were disintegrated, and the homogenates were centrifuged at 40,000g for 10 min. The 40,000g supernatant was further centrifuged at 48,000g for 30 min. The supernatant was amended with 0.1mM guanosine triphosphate (GTP) to stabilize carbendazim-binding activity of tubulin and immediately used for binding assays. All procedures were done at 4°C.

Binding assays and protein determination

One millilitre aliquots of the 48,000g supernatant of mycelial extracts were incubated with 10 µl of a methanolic solution containing the required amount of ^{14}C-carbendazim (specific activity 11.4 mCi/mmole, International Chemical and Nuclear Corp., U.S.A.) for 1 hr at 4°C. Binding of ^{14}C-carbendazim was examined by gel chromatography of the incubation mixture on a Sephadex G-25 column (25 x 1.5 cm) with PKMg solution as elution buffer. Fractions were collected and the radioactivity present in each was measured by liquid scintillation spectrometry. Radioactivity in protein fractions was considered to represent bound carbendazim.

Protein was determined according to the modified Lowry method (Hartree 1972) with bovine serum albumin as a standard.

Molecular weight (MW) determination of the carbendazim-protein complex

The molecular weight of the carbendazim-protein complex was determined by chromatography of the 48,000g supernatant of mycelial extracts incubated with ^{14}C-carbendazim (specific activity 4.5 mCi/mmole, supplied by Hoechst AG, West Germany) for 1 hr at 4°C on a Sephacryl S-200 column (25 x 1.5 cm). The following enzymes were used as markers: phosphoglycerate kinase (yeast), MW 34,000, malate dehydrogenase (*Candida utilis*), MW 60,000, lactate dehydrogenase (pig heart), MW 140,000, alcohol dehydrogenase (yeast), MW 148,000 and pyruvate kinase (pig heart), MW 237,000, respectively.

RESULTS AND DISCUSSION

Because of the slow growth of *Venturia* spp., the influence of culture age on maximum binding activity of mycelial extracts to ^{14}C-carbendazim was examined, using the sensitive isolate JS-18 as source of the extracts. After inoculation of mycelial fragments into a liquid medium, mycelia were harvested at one-week intervals for four weeks, followed by determination of the binding activity. As shown in Fig. 1, the ability of the cell-

free extracts to bind ^{14}C-carbendazim decreased with the age of the culture. In later experiments, therefore, one-week-old cultures were used to prepare mycelial extracts.

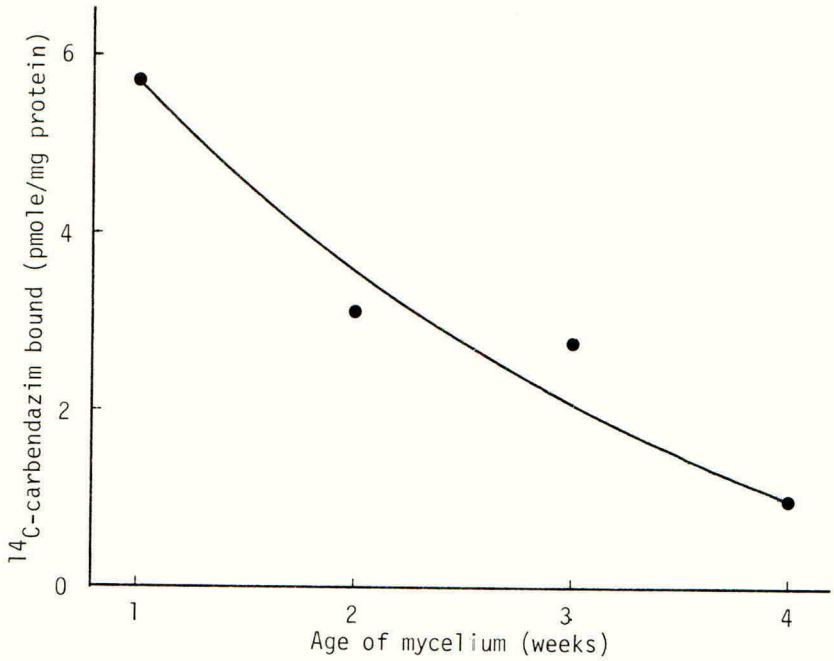

Fig. 1 Binding of ^{14}C-carbendazim to cell-free mycelial extracts from the benzimidazole-sensitive isolate of Venturia nashicola[a]

[a] Mycelial extracts were incubated with ^{14}C-carbendazim at 7.5 µM for 1 hr at 4°C. The quantity of bound carbendazim was determined with gel chromatography of the incubation mixture on a Sephadex G-25 column.

Binding data of the various experiments are given in Fig. 2. Recovery of the total radioactivity from the Sephadex column was higher than 99 %. Whereas extracts of the sensitive isolate bound ^{14}C-carbendazim in quantities between 0.6 and 2.4 % of the quantities added, the resistant isolates bound between 0.1 and 0.3 % of the quantities. Thus, the binding of cell-free extracts of carbendazim-resistant isolates was lower than that of the sensitive isolate, suggesting that decreased affinity of the binding sites to carbendazim might be involved in benzimidazole resistance in V. nashicola.

In V. nashicola, the manifestation of three different levels of resistance, viz. high resistance, intermediate resistance and weak resistance, could be attributed to three

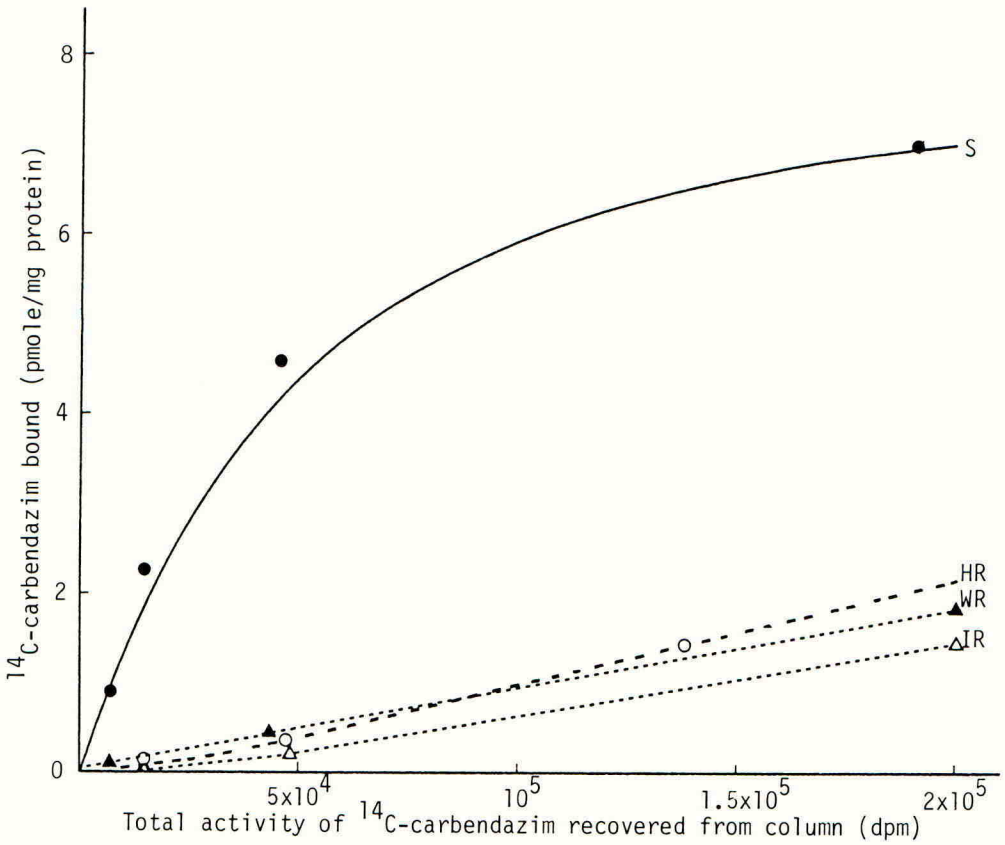

Fig. 2 Binding of ^{14}C-carbendazim to cell-free mycelial extracts from isolates of Venturia nashicola showing different levels of resistance[a] to this fungicide[b]
[a] HR, Highly resistant; IR, Intermediately resistant; WR, Weakly resistant; S, Sensitive.
[b] Mycelial extracts were incubated with ^{14}C-carbendazim at 7.5, 1.8, 0.6 and 0.3 μM, respectively, for 1 hr at 4°C. The quantity of bound carbendazim was determined with gel chromatography of the incubation mixture on a Sephadex G-25 column.

allelic mutations in a single gene (Ishii et al. 1984). Hence, it could be expected that the differences in resistance to carbendazim could be explained by the binding affinity. No difference, however, was detected in the binding activity to carbendazim between extracts of isolates with high, intermediate or weak resistance (Fig. 2).

Using gel chromatography on Sephacryl S-200 column, the labelled carbendazim-protein complex was eluted as a single peak. The elution volume was equivalent to a molecular weight for the labelled complex of approximately 100,000. This value is

similar to the molecular weight of tubulin in A. nidulans (Davidse and Flach 1977).

The results of this study suggest the possibility that the decreased affinity of tubulin-like protein to carbendazim is involved in the resistance. In addition, a gene conferring benzimidazole resistance may also encode tubulin production in V. nashicola. However, reduced uptake is also thought to contribute to benzimidazole resistance in Botrytis cinerea (Tripathi and Schlösser 1982). To clarify the mechanism of benzimidazole resistance, further characterization of the protein binding to carbendazim will be carried out. The possibility of the involvement of other mechanism(s) also will be checked. Contribution No.: Fruit Tree Res. Stn., A-190.

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SYNTHESIS OF SUGAR ALCOHOLS BY DICARBOXIMIDE-SENSITIVE AND -RESISTANT STRAINS OF *Neurospora crassa*

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ABSTRACT

A wild type (dicarboximide-sensitive) strain and a mutant (dicarboximide-resistant) *os-1* strain of *Neurospora crassa* were grown in synthetic media in the presence and absence of various osmotica. The two strains did not differ in their growth in media lacking osmoticum, but *os-1* grew much slower than wild type when subjected to osmotic stress by NaCl, KCl, glucose or fructose. When both strains were grown in media supplemented with 0.4 M NaCl, the glycerol content of their mycelia increased about 35 to 45-fold and the mannitol content about 5 to 10-fold, but the total amount of these sugar alcohols in *os-1* mycelia was approximately 30% lower than the total amount in wild type mycelia.

INTRODUCTION

Mutants of saprophytic and pathogenic fungi resistant to dicarboximide fungicides have been isolated under laboratory and field conditions (Beever & Byrde 1982; Leroux & Fritz 1984). This resistance is often associated with an increase in sensitivity to substrates of low water potential and a decrease in biological fitness (Grindle & Temple 1982; Beever 1983; Grindle 1983, 1984; Leroux & Fritz 1984). These features might explain why the occurrence of dicarboximide-resistant mutants in the field has not been associated with a loss of disease control (Beever & Byrde 1982).

Many fungi respond to environments of low water potential by an increase in the synthesis of intracellular solutes such as sugar alcohols which partly counterbalance the external water potential (Brown 1978; Wethered *et al.* 1985; Brown *et al.* 1986). This report describes experiments designed to test the hypothesis (Beever & Byrde 1982; Grindle 1984) that the abnormal osmotic sensitivity of dicarboximide-resistant mutants to environments of low water potential is due to an inability to accumulate sugar alcohols.

MATERIALS AND METHODS

Strains and media

The wild type strain 74-OR8-1a and the dicarboximide-resistant mutant *os-1* (allele P668) have been described by Grindle and Temple (1982, 1985). Cultures were grown on solid synthetic medium MM (Vogel 1964) with 15 g/l sucrose as carbon source to obtain conidia, and in liquid synthetic medium SC (Westergaard & Mitchell 1947) with 15 g/l glucose or fructose as carbon source for studies of growth and carbohydrate content. Glucose and fructose were filter-sterilised and added to the other medium components that had been autoclaved for 15 min at 120°C and 1.1 Pa. The SC medium

will be referred to as "basal medium"; it had a calculated water potential of -0.324 MPa.

Osmotica

The water potential of basal medium was reduced by -1.85 MPa by adding the required amounts of sugars, inorganic salts or polyethylene glycol (PEG 200). The amount of PEG 200 was determined experimentally using an osmometer, and the amounts of the other osmotica were obtained from the Handbook of Chemistry and Physics (Wolf & Brown 1967). The sugars were filter-sterilised and the other osmotica autoclaved before they were added to basal medium.

Growth studies

Conidia from 6-day-old cultures were suspended in deionised water, filtered through glass wool to remove mycelial fragments, counted with a haemocytometer and added to a large volume of liquid medium to give approximately 33 x 10^4 conidia/ml. Portions (150 ml) were dispensed into 250 ml flasks and incubated for 22 h at 26°C in darkness on an orbital shaker at 150 rev/min. The contents of each flask were harvested by centrifugation at 4°C for 10 min at 13000g and the mycelia dried by vacuum filtration as they were washed with 3 x 10 ml ice-cold basal medium. The vacuum-dried mycelial pads were blotted with Whatman 1 paper and weighed immediately to obtain fresh weights of mycelium per flask.

Carbohydrate studies

Mycelia, grown as described above, were harvested by rapid filtration on Whatman 1 paper in a Buchner funnel. The mycelial pads were left unwashed, as preliminary experiments showed that washing caused rapid and substantial leaching of intracellular solutes. Consequently, the mycelial pads were unavoidably contaminated with small amounts of spent culture medium.

Each mycelial pad was homogenised in 5 ml ethanol (80% v/v in deionised water), incubated for 30 min at 90°C, centrifuged at 1500g for 5 min, and the supernatant (ethanolic extract) retained. The process was repeated twice, and the three ethanolic extracts from each pad pooled for carbohydrate analysis. The remaining non-extractable material was dried at 105°C to obtain the "residual dry weight" of the mycelial pad.

Each pooled ethanolic extract was reduced in volume to 2 ml in a stream of warm air, after which 1 g resin (equal parts Amberlite IR-120[H] and IR-45[OH]) was added. The mixture was shaken for 15 min, centrifuged for 2 min at 1500g, and the supernatant retained. The resin was washed twice with 2 ml deionised water and the three supernatants pooled. Supernatants were evaporated to dryness in warm air, residues were dissolved in 0.55 ml anhydrous pyridine, and 0.3 ml hexamethyldisilizane and 0.15 ml trichloromethylsilane added to obtain trimethyl-silyl (TMS) derivatives. These were analysed using a Packard 437A gas chromatograph fitted with dual flame ionisation detectors. Aliquots (5 µl) were injected into a glass column containing 2% SE-52 stationary phase on Diatomite CLQ 100-120 mesh support, and derivatised carbohydrates separated using a temperature programme of 100-260°C increasing at 4°C/min. The nitrogen carrier gas flow rate was 20 ml/min and the hydrogen and air flow rates were 20 and 200 ml/min, respectively. The injection and detector temperatures were 250°C and 270°C, respectively. Standard carbohydrates were run before and after a batch of samples. Carbohydrates in the samples were identified by co-chromatography and

verified on a second glass column packed with 2% OV-17 on Diatomite CLQ 100-120 mesh support. Operating conditions were identical to the first column except that the final temperature was held for 10 min. Quantification was by external standard and one-point calibration method using a Shimadzu Chromatopac C-R3A data processor.

RESULTS

Wild type and mutant strains grew equally well in basal medium lacking osmoticum, although growth was greater with glucose than fructose as carbon source (Table 1). All osmotica caused a similar reduction in growth of the wild type, irrespective of the carbon source (range: 19-42%; mean: 34%). NaCl, KCl, glucose and fructose caused a much greater reduction in the growth of os-1 (range: 85-95%; mean: 90%) than did PEG 200 and glycerol (range: 33-58%; mean: 42%).

Wild type and mutant strains did not differ substantially in their ability to synthesise sugar alcohols (Table 2). The two strains produced similar amounts of the individual polyols when grown in the absence of osmoticum, the predominant sugar alcohol being glycerol with glucose as the carbon source and mannitol with fructose as the carbon source. In the presence of osmoticum, the pool sizes of glycerol and mannitol increased in both strains but the concentrations of other polyols showed little change. With glucose as carbon source, the glycerol content of wild type and mutant increased 45-fold and 37-fold, respectively. The increase in mannitol content was less marked (12-fold in the wild type and 10-fold in os-1). A similar pattern was obtained with fructose as the carbon source. The combined amounts of these two sugar alcohols were approximately 30% lower in the mutant than in the wild type.

The wild type and os-1 strains differed consistently in their trehalose content. In the absence of osmoticum, the wild type produced twice as much trehalose as the mutant. In the presence of osmoticum, the trehalose content of the wild type did not alter significantly ($P > 0.05$) but there was a four-fold increase in the mutant.

The solute potentials generated by glycerol and mannitol were calculated using Van't Hoff's equation (Nobel 1983). The concentrations of these sugar alcohols within the mycelia were determined on the basis that the residual dry weight of mycelium was 12.5% of the fresh weight and that 50% of the fresh weight was cytoplasmic water. Accordingly, when mycelia were grown in 0.4 M NaCl with glucose as carbon source, the solute potential attributable to glycerol and mannitol was -0.662 MPa in the wild type and -0.424 MPa in the mutant. As the water potential of the basal medium supplemented with 0.4 M NaCl was calculated to be -2.17 MPa, the solute potential generated by glycerol and mannitol was therefore equal to only 31% of the external water potential in the wild type and 20% in the mutant. Similar values were obtained with fructose as the carbon source.

DISCUSSION

Although the dicarboximide-resistant os-1 mutant is usually more sensitive than the wild type to media of low water potential, the type of osmoticum used to lower the water potential has a marked effect on the degree of "osmotic sensitivity". Ionic osmotica such as NaCl and KCl, and hexoses such as glucose and fructose, have a far greater inhibitory effect than either glycerol or PEG 200 on the growth of os-1 (Table 1). Beever

TABLE 1

Effect of various osmotica on growth[a] of a wild type and a dicarboximide-resistant os-1 mutant of Neurospora crassa

Growth medium		Fresh wt. mycelia[c]	
carbon source	osmoticum[b]	wild type	os-1
glucose	basal medium	100[d]	100[e]
"	NaCl	67 (2)	10 (2)
"	KCl	64 (1)	15 (1)
"	glucose	76 (1)	5 (1)
"	PEG 200	58 (2)	42 (3)
"	glycerol	81 (5)	65 (1)
fructose	basal medium	100[f]	100[g]
"	NaCl	64 (2)	10 (1)
"	KCl	63 (2)	11 (3)
"	fructose	59 (4)	6 (1)
"	PEG 200	64 (5)	67 (4)

a Growth measured as fresh weight of mycelia after 22h at 26°C in liquid medium with 15 g/l glucose or fructose as carbon source.

b The water potential of the basal medium was -0.324 MPa, and each osmoticum reduced this by -1.85 MPa (equivalent to 0.4 M NaCl).

c Expressed as % of growth in basal medium, means and (S.E.M.) of 4 replicates.

d 100 = 695 (70) mg fresh weight mycelium per flask, mean and (S.E.M.) of 12 replicates.

e 100 = 692 (73) mg per flask, as above.

f 100 = 335 (20) mg per flask, as above.

g 100 = 297 (20) mg per flask, as above.

TABLE 2

Effect of NaCl as osmoticum on the carbohydrate content of a wild type and a dicarboximide-resistant os-1 mutant of Neurospora crassa

Strain	Growth medium		Carbohydrate content of mycelia[a]					
	Carbon source (15 g/l)	NaCl[b] (0.4 M)	Arabitol	Erythritol	Glycerol	Inositol	Mannitol	Trehalose
wild	glucose	-	3.1 (0.2)	1.0 (0.2)	21 (3)	2.3 (0.2)	9 (2)	73 (12)
os-1	"	-	2.1 (0.1)	0.4 (0.2)	17 (7)	1.6 (0.3)	7 (2)	32 (5)
wild	"	+	4.4 (0.1)	3.2 (1.6)	960 (87)	2.0 (0.1)	109 (7)	54 (3)
os-1	"	+	2.5 (0.1)	1.1 (0.3)	640 (62)	1.4 (0.1)	65 (5)	125 (20)
wild	fructose	-	2.8 (0.2)	3.4 (0.2)	14 (3)	1.3 (0.1)	78 (5)	25 (3)
os-1	"	-	2.5 (0.3)	1.7 (0.1)	14 (3)	0.6 (0.1)	46 (2)	13 (1)
wild	"	+	3.3 (0.2)	3.5 (0.2)	609 (36)	2.8 (0.1)	330 (22)	29 (4)
os-1	"	+	1.4 (0.1)	3.0 (0.3)	456 (46)	1.8 (0.1)	202 (17)	52 (4)

a Expressed as nmole/mg residual dry weight of mycelia grown for 22h at 26°C in liquid medium. Values are means and (S.E.M.) of 6 replicates.

b - medium lacking NaCl; + medium containing NaCl.

(1983) also showed that glycerol was less effective than NaCl, KCl or glucose in reducing radial growth of dicarboximide-resistant mutants on solid media. These differences among osmotica may reflect differences in toxicity: high concentrations of some compounds, such as NaCl, could have both toxic and osmotic effects on growth whereas other compounds, such as glycerol, could be much less toxic. For example high concentrations of glycerol are less inhibitory to enzymes than many other solutes (Brown 1978). The possible toxic influence of high concentrations of inorganic solutes on fungal metabolism have been commented on by Eamus and Jennings (1986). The different effects of the osmotica on growth of the two strains could be due to differences in membrane permeability; for example, that of the mutant might allow more ready access of toxic ions to the cytoplasm.

The data in Table 2 clearly demonstrate that os-1 does not lack the capacity to synthesise more sugar alcohols in response to osmotic stress. In both os-1 and wild type glycerol and, to a lesser extent, mannitol act as solutes which compensate for the low water potential of their growth media. However, calculations showed that these two polyols represented only 20-30% of the solute potential of the mycelium required to counterbalance completely the water potential of the medium. Therefore, other solutes must play an important role in the adaptation of N. crassa to environments of low water potential. Similar conclusions with respect to other fungi have been reached by Brown et al. (1986) and Eamus and Jennings (1986).

Differences in the trehalose content of wild type and mutant are difficult to explain. Accumulation of trehalose in fungi is usually associated with periods of reduced growth (Thevelein 1984) and so the increase in trehalose content of os-1 grown in the presence of 0.4 M NaCl could be a consequence of the marked reduction in its growth. However, this explanation cannot account for the different trehalose contents of the two strains when grown in the absence of NaCl. Under such conditions the strains have similar growth rates but os-1 mycelia contain only half as much trehalose as wild type mycelia.

It is evident from this study that the abnormal sensitivity of the dicarboximide-resistant os-1 mutant of N. crassa to media of low water potential is not due to an inability to accumulate sugar alcohols. The greater "osmotic sensitivity" of os-1 to ionic osmotica suggests that the biochemical lesions caused by the dicarboximide-resistance os-1 gene influence the properties of the cell membrane. Further work is in progress to determine whether the structural and chemical composition of the os-1 membrane is altered, and whether influx and efflux of solutes is abnormal.

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PYRETHROID RESISTANCE IN THE COTTON BOLLWORM, HELIOTHIS ARMIGERA - A CASE HISTORY FROM THAILAND

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ABSTRACT

Following reports of pyrethroid field failures in cotton in Thailand during 1984, a resistance monitoring programme was instigated by a group of pyrethroid manufacturers and the Thai Department of Agriculture. Three cotton growing areas were selected on the basis of past pyrethroid performance, and sampled through the cropping season. Although all populations were heterogeneous, the mean level of pyrethroid resistance at the beginning of 1985 had not declined significantly from that of the last pyrethroid-treated population of 1984. Insects from all sites were similar with respect to pyrethroid tolerance and in the laboratory, cross or multiple-resistance to DDT and the carbamates was detected. When considering cropping practices and the ecology of the cotton bollworm (Heliothis armigera) in Thailand, the technical prospects for resistance management seem favourable. In practice however, a favourable outcome for the goverment's proposed alternation strategy will be dependent upon successful farmer re-education and improved control of pyrethroid usage.

INTRODUCTION

Following indications of reduced efficacy of the pyrethroid insecticides against the cotton bollworm, Heliothis armigera in Thailand, an initiative was set up in September 1984, by the I.C.I. Asiatic (Agriculture) Company (I.A.A.C) in conjunction with the Thai Department of Agriculture to carry out a preliminary investigation of the situation.

This study, and similar findings by Shell Research Limited led to the Pyrethroid Efficacy Group (P.E.G.) and the Thai Department of Agriculture agreeing to co-operative research in 1985 to confirm and further define the resistance problem.

Although there has been no continuous monitoring of H.armigera susceptibility to pyrethroids in the laboratory, Thai government field trials have indicated a change in H.armigera susceptibility to pyrethroids in Tak Fa (TakLi) over several years (Wangboonkong, 1981) (Table 1).

TABLE 1

Changes in the efficacy of pyrethroids applied to cotton in Tak Fa for the control of H.armigera between 1977 and 1983 (Data courtesy of the Nakonsawan Field Crops Research Station).

	CHEMICAL TREATMENT					
	FENVALERATE		CYPERMETHRIN		DELTAMETHRIN	
YEAR	DOSAGE (g ai/ha)	% control	DOSAGE (g ai/ha)	% control	DOSAGE (g ai/ha)	% control
1977	50	83.45	50	83.22	-	-
1978	50	71.43	50	64.94	6.25	75.96
1979	50	39.13	50	40.05	7.5	31.81
1980	50	71.90	50	71.90	7.5	71.90
1981	100	36.41	50	52.31	9.0	48.21
1982	100	-	50	-	12.5	-
1983	100	22.55	50	9.82	12.5	10.55

Most farmers in upland cropping areas of Thailand cultivate small areas of land (1-2 ha) on which they grow two main crops per year. The first crop is predominantly maize followed by either sorghum, mung - bean, soyabean, black matpe or cotton. Maize is the major crop in terms of area grown (Table 2).

Table 2 Crop production in Thailand for the planting seasons of 1983-1985.

	Area Harvested (million ha)		
Crop	Early Monsoon April-May	Mid Monsoon August	Post Monsoon November - early December
Maize	1.49	0.066	-
Sorghum	?	0.133	-
Cotton	-	0.080	-
Soyabean	0.097		0.036
Mungbean	0.069	0.302	0.093
Black matpe	0.013	0.058	0.018
	2.308		0.147

Sources of food for the cotton bollworm in Thailand include both cultivated crops and wild host plants. Maize, cotton, soyabean, black matpe, sorghum and mungbean are the major crops infested. The bollworms are also found on minor crops and various shrubs, herbaceous plants and weeds, such as Ageratum conyzoides, Veronica cinerea, Physalis minima, and Datula metel.

1-2 generations occur on maize starting in April, before cotton becomes attractive, and generally 2-3 generations occur in cotton with peaks in August, September and October. It is suspected that 2 generations are produced in sorghum which is harvested somewhat later than cotton and then 1-2 generations occur on wild hosts. This probably equates to 6-8 generations per year, but generation overlap makes accurate determination difficult.

Within the cropping rotation, only cotton receives an intensive insecticide input. Until 1980, DDT and DDT/toxaphene were widely used. These products were quickly replaced by the pyrethroids following reports of reduced efficacy and a growing perception of the environmental hazards associated with DDT, and also because the pyrethroids were relatively cheap. In recent years, pyrethroids have been the dominant insecticides used for the control of H.armigera.

Government recommendations are to spray for bollworms at a threshold of 15-20 larvae per 100 plants. In practice farmers spray at squaring or when (medium to large) larvae are visible in the crop. Sprays are then applied weekly, switching to twice weekly when pest pressure is heavy. Twenty sprays per season are not uncommon.

Other chemicals used in cotton include carbamates, monocrotophos and dimethoate, used for sucking pest control during the early season in cotton and also in mungbean, soybean, etc. Insecticide use in other crops is minimal, on an "as needed" basis, eg. methomyl used to protect sorghum heads from H.armigera damage. In many instances, particularly for maize, crop value is low and does not warrant treatment, even of high bollworm infestations. So only a proportion of the H.armigera population receives pyrethroid selection pressure, for 2-3 out of a possible 6-8 generations, and maize provides a large reservoir of H.armigera which migrate to alternative hosts when the crop is harvested.

METHODS

A simple dip test was adopted as the means of detecting and monitoring pyrethroid resistance in the field (Watkinson et al., 1984). The test is relatively robust and can be performed with the minimum of equipment in contrast with topical application techniques. In Thailand it proved possible to collect large numbers of the third instar larvae required for the test directly from the field, avoiding the need for rearing facilities. Sampled populations were thus those surviving previous insecticide treatment.

Because susceptible base-line data were unavailable, attempts were made to include sampling sites thought likely to yield susceptible populations of H.armigera. The characterisation of a susceptible population could then be used to define a discriminating dose for further monitoring. In 1985 three sites were chosen:

i) Tak Fa - A large cotton growing area in which farmers had recently obtained poor control of H.armigera with pyrethroids.

ii) Lamnarai - A large cotton growing area where farmers had not previously experienced poor control from pyrethroids.

iii) Pakchong - A relatively small area of cotton, thought to be a suitable site for the collection of susceptible H.armigera, especially from maize, in the absence of pyrethroid selection pressure for several generations.

The sampling programme involved the collection of a minimum of 100 third instar larvae on three successive days from each of the geographic regions. This programme was repeated through the season to encompass an initial generation in maize followed by two generations in cotton, thus monitoring spatial and temporal changes in pyrethroid susceptibility. Cypermethrin (25% EC) was chosen as the standard test chemical. Further studies in the laboratory involved the use of standard topical application techniques (Anon., 1970).

RESULTS

Extremely high concentrations of cypermethrin were needed to kill the most tolerant insects. Initial bioassays on insects collected from maize in 1985 gave similar results to the bioassays from cotton in the previous year. The populations were heterogeneous (dose response covering 4 orders of magnitude) but similar at all 3 sample sites. Thus the field assays in maize did not provide a susceptible baseline from which to define a discriminating dose. Subsequent assays of insects collected from cotton did not result in substantially more tolerant or more homogeneous strains, with the possible exception of Pakchong, despite widespread pyrethroid use (Table 3).

TABLE 3

The susceptibility of H.armigera from three sites in Thailand to cypermethrin

	Bioassay results (ppm)*					
Sample collection	Pakchong		Lamnarai		Tak Fa	
	LC50	LC90	LC50	LC90	LC50	LC90
Cotton, Oct. 1984	-	-	-	-	330	780
Corn, Jul/Aug. 1985	146	2160	163	2150	228	1040
Cotton, Aug/Sept. 1985	261	1220	150	1110	235	2750
Cotton, Sept/Oct. 1985	219	1820	125	1690	230	1420

* assessed 24h after dipping of 3rd instar larvae. Regression analysis was not performed as it is inappropriate for the treatment of dose-response curves of heterogeneous populations. Approximate LC values have been estimated by eye from probit plots.

In order to supplement these studies, and to investigate patterns of cross-resistance opposite potential alternation strategies, pupae were collected at Tak Fa and sent to the University of Reading where a culture of resistant insects was successfully established. Comparison with a laboratory susceptible strain demonstrated a 50-100x resistance factor for the pyrethroids. A similar level of resistance to DDT was apparent. It is not clear whether the mechanism(s) involved were selected due to previous DDT use or current pyrethroid use.

Significant resistance to endosulfan or organophosphates was not detected, but resistance to a carbamate was observed (Table 4).

TABLE 4

Bioassay of susceptible (Oxford) and resistant (Thailand) strains of H.armigera treated with various insecticides at instar III.[+]

INSECTICIDE	LD50 SUSCEPTIBLE	LD50 RESISTANT	RESISTANCE RATIO
cis-cypermethrin	0.009	0.92	102
trans-cypermethrin	0.031	2.47	80
fenvalerate	0.015	0.77	51
DDT	0.60	75.24	124
carbaryl	4.01	110.28	27.5
endosulphan	1.24	2.30	1.86
monocrotophos	1.48	1.50	1.01
diazinon	0.72	3.88	1.80

[+] Topical application, µg/larvae (30-40mg) applied in 1 µl acetone

Limited studies with synergists and neurobiological assays gave an indication of multiple resistance mechanisms, eg. increased metabolism and nerve insensitivity.

It was observed that the resistant strain had lost tolerance since its introduction to the laboratory, and that a pressured sub-culture was less fecund and developed more slowly than the laboratory susceptible strain (A. McCaffery, pers. comm.).

CONCLUSIONS AND STRATEGY

Given the similarity of insect susceptibility throughout the cotton growing regions of Thailand, it is surprising that the farming community still perceives "hot-spots" of pyrethroid resistance. Variability in pest pressure is strongly implicated - a reminder that the demonstration of increased tolerance in the laboratory is not necessarily predictive of field resistance leading to control failure.

In response to pyrethroid resistance, aided by the findings of the PEG study, the Thai Department of Agriculture has produced a strategy of recommended alternations to reduce selection pressure. Rotation groupings comprise the organophosphates, pyrethroid mixtures, the carbamates, an insect growth regulator (chlorfluazuron) and the pyrethroids.

In 1984 many Thai farmers persisted with the pyrethroids in the face of field failure. In 1985, more farmers adopted the alternation strategy; however some problems with the strategy remain. Firstly, farmers may alternate different pyrethroid brands through ignorance of the theory underlying the strategy. Secondly, local dealers may have a limited supply of alternative chemicals and will offer discounts to increase pyrethroid sales. Thirdly, the alternation is haphazard rather than co-ordinated and finally, many Thai farmers need further training in crop protection practices to improve their ability to manage pesticide use effectively.

Because of the diversity of the Thai insecticide market (over 20 brands of pyrethroid, many locally produced) it would be difficult to instigate a co-operative programme involving Industry and the Government (as in Australia) to control the use of pyrethroids. There is however, a natural check on insecticide usage and selection pressure. Following years of severe infestation, a number of Thai farmers have abandoned cotton growing on economic grounds in favour of 'low input' crops. There has been an overall reduction in the area of cotton grown in Thailand over recent years (Table 5).

TABLE 5

Cotton Production in Thailand 1980-1985 (Data courtesy of the Nakonsawan Field Crops Research Centre).

YEAR	HARVESTED AREA (ha)	PRODUCTION (tons)	YIELD (kg/ha)
1980	145,581	192,570	1,331
1981	152,302	175,733	1,156
1982	107,141	122,008	1,138
1983	100,009	119,225	1,194
1984	70,400	83,000	1,188
1985	43,520	N/A	N/A

Cotton production has reduced most in those areas where pest problems are most severe, eg. Tak Fa. This solution is of course less than satisfactory since Thailand is keen to protect its cotton industry. The best hope for the future is continued cooperation between the Department of Agriculture and the insecticide manufacturers, to ensure that the management of resistance is improved, with an emphasis on programmes of farmer re-education.

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STUDIES ON RESISTANCE TO INSECTICIDES IN THE COTTON BOLLWORM HELIOTHIS ARMIGERA WITH SPECIAL REFERENCE TO THE PYRETHROIDS

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ABSTRACT

A strain of Heliothis armigera resistant to insecticides was obtained from a cotton growing area of Thailand. Bioassay with a range of insecticides revealed up to 100-fold resistance to pyrethroids, 125-fold resistance to DDT and 27-fold resistance to carbaryl when compared to a susceptible strain. Assays with synergists suggested the presence of both oxidase and esterase detoxication mechanisms in the susceptible strain; synergism was greater in the R strain with trans cypermethrin, though less with the mixture of cis isomers. Pharmacokinetic studies on the S strain with 14C-trans cypermethrin showed rapid penetration and early appearance of products of ester bond cleavage in the excreta. Transitory oxidative products also appeared. Neurophysiological studies indicated that the resistant strain might have some nerve insensitivity, which could account for cross-resistance to DDT.

INTRODUCTION

The noctuid Heliothis armigera, known locally as the cotton bollworm, American bollworm or gram pod borer, occurs throughout much of Africa, Asia and Australia and is one of the world's most important pre-harvest pest insects (Reed & Pawar 1982). The larvae of this insect are known to feed on over 160 species of plant of which over 70 are cultivated crops including cotton, cereals, vegetables and legumes.

A wide variety of insecticides has been used to control the larvae and in many areas the applications have been heavy and frequent. The species has therefore been subjected to considerable selection pressure with the result that resistance has developed to all the major classes of insecticides in those areas where they have been used (Wolfenbarger et al. 1981). The overall pattern of this development of resistance to insecticides in Heliothis armigera is similar to that of many other insects. First resistance to DDT and DDT + toxaphene followed by endosulphan and endrin developed throughout much of its range (e.g. Wangboonkong 1971, Wilson 1974, Goodyer et al. 1975, Kay 1977, Basson et al. 1979, Wolfenbarger et al. 1981). Resistance to methyl parathion in Australia (Wilson 1974) and to carbaryl in Thailand soon followed.

Resistance to the synthetic pyrethroids, leading to complete field failure, is a recent phenomenon which has been documented for Australia

(Gunning et al. 1984) and which has occurred rapidly following the introduction of these materials. Simultaneous reports of pyrethroid failures against Heliothis armigera appeared from cotton growing areas of Thailand and Turkey from 1983 (M.D. Collins, personal communication) but published details are not available. One of the aims of these studies has been to assess the status of resistance in a strain of Heliothis armigera collected from a cotton growing area of Thailand.

In order to understand the basis of resistance to pyrethroids in this insect we have begun studies on the mechanisms involved. Synergism and pharmacokinetic studies using radiolabelled trans cypermethrin have been used cautiously to look for evidence of metabolic resistance (Sawicki 1975). Resistance to the pyrethroids is frequently associated with insensitivity in the target site nerves (Miller et al., 1979) and we have begun studies to examine this.

MATERIALS AND METHODS

Insects

A strain of Heliothis armigera reported to be resistant to insecticides was obtained as larvae from a cotton growing area at Tak Fa in Thailand. Adults emerging from the resulting pupae were allowed to breed. Breeding stocks of the insects were continually subjected to a selection of 0.025µg of cis cypermethrin. The bioassay and synergism studies described here were performed largely on the F1 and F2 generations and the neurophysiological studies on F3 and F4 insects. A strain of the insects susceptible to insecticides was maintained in the laboratory from stock originally obtained from the Institute of Virology in Oxford. All the insects were maintained at 25°C under standard conditions and fed on a bean-based artificial diet (Ahmad & McCaffery 1986).

Chemicals

Technical grade insecticides and synergists were obtained from manufacturers as detailed previously (Ahmad & McCaffery 1986, unpublished data).

Insecticide Bioassay

Third instar larvae of standard weight (30-40mg) were topically dosed on the dorsal thorax with a 1µl drop of insecticide in acetone (Ahmad &McCaffery 1986) based on recommended methods (Anon, 1970). A range of serial dilutions for each insecticide with each strain was made following range-finder tests. Control insects were treated with acetone alone. A minimum of 40 insects were tested at each dose. Mortality was determined 72h after treatment. Probit analysis (Finney 1971) was performed on the results using an SAS analysis package on an Amdahl mainframe computer.

Neurophysiological studies

In preliminary experiments third instar larvae were topically treated with 12.5 ng of cis cypermethrin as above. The larvae were decapitated at known times after treatment, the nerve cord exposed by dorsal dissection and the preparation bathed in a balanced salt solution. The multiunit activity (MUA) of the nerve cord was monitored by means of a suction electrode connected to an appropriate amplification and conditioning system (Neurolog, Digitimer Ltd.) and recorded on magnetic tape for later analysis. Recordings of MUA from both susceptible and resistant larvae were made. Action potentials with

a signal:noise ratio exceeding 2:1 were counted for successive 5s intervals and the mean frequency used as a measure of spontaneous nervous activity.

Synergism studies

Thirty minutes prior to treatment with insecticide larvae were topically treated with standard doses of 20µg of piperonyl butoxide (PB), 20µg of S,S,S-tributyl phosphorotrithioate (DEF), a combination of 20µg of PB and 20µg of DEF, or 10µg of 2,2,2-trifluoro-1,1-bis (4-chlorophenyl) ethanol (FDMC). These doses were determined in preliminary toxicity and inhibition studies. Insecticide bioassays were then performed as described above.

Pharmacokinetics and metabolism

^{14}C-cyclopropyl 1RS trans RS cypermethrin (9.6 µCi/mg and 99% purity) was supplied by Shell Research Ltd. Authentic metabolites were prepared or obtained as described in Lee et al. (1986). 1.0µl aliquots of 0.075µg of ^{14}C trans cypermethrin in acetone were topically applied as above to 15 sixth instar larvae. The insects were given food and kept for various times up to 48 h before extraction.

Larvae were surface washed 3 times with 3 ml of acetone, the combined washes centrifuged, reduced in volume and aliquots taken for scintillation counting (Lee et al., 1986). Excreta were extracted three times in methanol, centrifuged, aliquots taken for counting and the samples dried and analysed by TLC (Lee et al., 1986). Larvae were homogenised in methanol, extracted three times, centrifuged, aliquots taken for counting, dried and analysed as above.

Areas of radioactivity on thin-layer plates were determined using a Panax scanner, scraped from the plates and extracted with acetone. Aliquots of acetone extracts were taken for scintillation counting. Metabolites were identified by comparison with radiolabelled standards run on the same plates (Lee et al., 1986).

RESULTS

Toxicology

The Thai strain showed considerable resistance to the pyrethroids with LD_{50} ratios of between 50 and 100 when compared to the susceptible strain (Table 1). The insects also showed 125-fold resistance to DDT and 27-fold resistance to carbaryl. There was no resistance to endosulfan or the two organophosphates.

Neurophysiology

Exposure to 12.5ng cis cypermethrin immobilised larvae of the susceptible strain within 15 min. but did not immobilise larvae of the resistant strain. Spontaneous activity was undetectable in the nerve cord of treated susceptible larvae except in one larva (Table 2). The spontaneous activity of larvae of the resistant strain was not reduced by exposure to cypermethrin.

Synergism

PB synergised all of the insecticides tested in the susceptible strain except cis cypermethrin and DDT (Table 3). Synergist ratios of between 1.3 and 1.7 were obtained with the trans cypermethrin, fenvalerate and monocrotophos. Endosulfan, carbaryl and diazinon were moderately synergised by PB but it antagonised DDT (Table 3). DEF

TABLE 1

LD_{50} values (μg/larva) for susceptible (Reading) and resistant (Thailand) strains of Heliothis armigera treated with various insecticides during 3rd instar (30-40mg)

Insecticide	Susceptible LD_{50}	S.E.	Resistant LD_{50}	S.E.	Resistance Ratio (R/S)
cis Cypermethrin	0.009	0.13	0.92	0.10	100
trans Cypermethrin	0.031	0.15	2.5	0.06	80
Fenvalerate	0.015	0.22	0.77	0.09	51
DDT	0.60	0.37	75	0.14	130
Endosulfan	1.2	0.24	2.3	0.14	1.9
Carbaryl	4.0	0.76	110	0.10	28
Diazinon	2.2	0.40	5.9	0.17	2.7
Monocrotophos	1.1	0.08	1.50	0.09	1.4

Data condensed from Ahmad & McCaffery (1986).

TABLE 2

Frequency of action potentials in the ventral nerve cord of susceptible and resistant strains of Heliothis armigera

	Frequency of nerve action potentials (Hz) in individual: susceptible larvae mean (range)	resistant larvae mean (range)
Untreated	14 (6 - 17)	12 (5 - 14)
Treated, 15-30min exposure	0, 10 (5 - 13)	10 (3 - 17)
Treated, >45min exposure	0, 0, 0, 0	11 (5 - 13), 18 (14 - 20), 14 (7 - 19)

synergised the action of the pyrethroids and carbaryl but had no effect on organophosphate action. When applied before trans cypermethrin or fenvalerate increased synergism was obtained using a combination of PB + DEF over and above the synergistic effects of each component alone. FDMC did not synergise the action of DDT.

Both PB and DEF were potent synergists for trans cypermethrin in the Thai resistant strain (Table 4). Only slight synergism of cis cypermethrin was obtained with DEF.

Pharmacokinetics

Initial studies with 14C-trans cypermethrin were carried out with susceptible insects to establish methods for use with resistant strains. First the penetration, metabolism and excretion of the material was examined in 6th instar insects. Uptake was rapid and label quickly

TABLE 3

Synergism by PB, DEF and FDMC of topically applied insecticides to 3rd instar susceptible Heliothis armigera

Insecticide	Synergist Ratio[1] (at LD_{50}) with: PB	DEF	PB+DEF	FDMC
cis Cypermethrin	0.99	6.0	-	-
trans Cypermethrin	1.5	4.8	12	-
Fenvalerate	1.7	2.5	26	-
DDT	0.58	-	-	1.0
Endosulfan	2.2	-	-	-
Carbaryl	3.4	3.2	8.3	-
Diazinon	2.3	1.1	2.2	-
Monocrotophos	1.3	1.1	1.2	-

[1] LD_{50} for insecticide alone ÷ LD_{50} for insecticide + synergist.

TABLE 4

Synergism by PB and DEF of topically applied insecticides to 3rd instar of resistant Heliothis armigera

Insecticide	Synergist Ratio[1] (at LD50) with: PB	DEF
cis Cypermethrin	Not tested	2.1
trans Cypermethrin	6.5	9.4

[1] LD_{50} for insecticide alone ÷ LD_{50} for insecticide + synergist.

appeared in the excreta (Table 5).

Unmetabolised substrate accounted for most of the radioactivity recovered at 2h in the surface rinses but in the longer term most of the radioactivity in the insect was in the form of metabolites. The most abundant was the acid (t-$C1_2$ CA). Other major components were identified as 4-OH trans-cypermethrin and the hydroxy acid (t-OH-Cl_2CA and 'conjugates'. All increased in concentration in the excreta except 4-OH trans cypermethrin (Table 6). Conjugates were hydrolysed by reflexing with 0.02M methanolic KOH in hexane and the major component was again identified as t-CL_2 CA.

DISCUSSION

Comparison of insecticide bioassay data from our susceptible strain of Heliothis armigera with those from other susceptible strains from various localities indicates that this reference strain is not unnaturally susceptible to any of the materials used in these studies (Ahmad & McCaffery, 1986). Apart from values for mixed cis and mixed

TABLE 5

Distribution of radioactivity in surface rinses, larval bodies and excreta of 6th instar susceptible Heliothis armigera at various times after topical application of 14C trans cypermethrin

Time after application (h)	Percentage of applied radioactivity in: surface rinses	larval bodies	excreta	Total recovery
2	52	27	12	91
4	33	29	22	84
8	25	35	24	84
24	12	34	28	74
48	4	12	43	59

TABLE 6

Metabolites in excreta of 6th instar susceptible Heliothis armigera following topical application of 14C-trans cypermethrin.

Time after application (h)	Percentage of applied radioactivity as: t-HO-Cl_2CA	4'-HO-cyp	Cl_2CA	Conjugates	Other	Total
2	1	1	6	1	3	9
4	2	3	8	1	8	14
8	2	8	8	2	4	20
24	3	4	12	3	6	22
48	6	6	18	6	7	36

trans isomers of cypermethrin and for diazinon which have not been reported previously the LD_{50} values obtained here are almost identical to those of other studies for DDT (Whitlock 1973, Wilson 1974), endosulfan (Kay 1977) and carbaryl (Deema et al. 1974), slightly higher than others for DDT (Brader 1968), endosulfan (Brader 1968) and monocrotophos (Goodyer et al. 1975) and slightly lower for fenvalerate (Gunning et al. 1984). The Reading strain reflects a level of susceptibility likely to be found in unselected field populations.

The present studies indicate a high level of resistance in the Thai strain to the two pyrethroids tested. The resistance ratio (102) to the cis isomers of cypermethrin was considerably higher than to the trans isomers (80) whilst the value for fenvalerate was lower (51). Slopes of regression lines were lower for these materials in the resistant strain than in the susceptible strain, suggesting heterogeneity in the former (Ahmad & McCaffery 1986).

Resistance to DDT was especially high (125-fold) and we were unable to achieve more than 50% mortality with the highest doses which could be applied. DDT has not been used in Thailand for some time and the results are strongly suggestive of a cross-resistance between the synthetic pyrethroids and DDT. Such patterns of cross resistance are well known in houseflies (e.g. De Vries & Georghiou 1981), in which the kdr mechanism confers broad cross-resistance to both the pyrethroids and DDT by way of decreased nerve sensitivity. The results of the neurophysiological studies suggest that Heliothis armigera might possess a nerve insensitivity of this type. Spontaneous activity in the nerve cord of cypermethrin treated susceptible larvae was undetectable whereas that of resistant insects was not reduced by exposure to the insecticide.

The results of the synergism studies suggest that both oxidative and esteratic enzymes are involved in the detoxication of topically applied insecticides in this insect and that in the susceptible strain co-administration of both PB and DEF leads to an especially elevated synergist ratio. The results of the pharmacokinetic studies with ^{14}C-trans cypermethrin show that the major radiolabelled products are either the acid or the hydroxy acid, both of which are products of ester bond cleavage and would be expected as a result of esterase attack. It is not clear why 4-OH trans-cypermethrin tends to reach a maximum level in this insect at 6-8h and then remains more or less constant or falls whereas other metabolites continue to increase. With the resistant strain both PB and DEF are effective synergists, but we have not yet been able to examine the pharmacokinetics of the resistant insects.

These studies indicate that this strain of Heliothis armigera from Thailand is highly resistant to the synthetic pyrethroids and DDT and has moderate resistance to carbaryl. However, whether the mechanisms responsible for this pattern of resistance include nerve insensitivity, esteratic and oxidative biochemical resistances, or both remains unclear. Further studies on these mechanisms and their inheritance are in progress.

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TREATMENT OF A FARM GRAIN STORE WITH PIRIMIPHOS-METHYL AND THE CONSEQUENCES FOR RESISTANCE IN SAW-TOOTHED GRAIN BEETLES (ORYZAEPHILUS SURINAMENSIS)

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ABSTRACT

Saw-toothed Grain Beetles (Oryzaephilus surinamensis) have been present on a farm in North Yorkshire since 1972 and a range of pesticides including malathion, pirimiphos-methyl and phosphine have been used to kill insects in the grain store facilities. In 1984 and 1985 samples of O. surinamensis were collected from the farm and found to be resistant when tested with discriminating doses of malathion or pirimiphos-methyl. Responses ranged from 30-50% KD (knockdown) for pirimiphos-methyl, depending upon the sampling site. Following treatment of interior surfaces of the grain stores with pirimiphos-methyl wettable powder in June 1985, insects were collected and tested with a discriminating dose of pirimiphos-methyl. Insects from an untreated animal house gave 51% KD whereas those collected from the treated barley store gave only between 13 and 25% KD. Subsequently the farmer treated the 1985 barley crop with pirimiphos-methyl and some survivors of this treatment gave only 2% KD at the discriminating dose. Dose-response lines for the animal house and barley store populations confirm that selection has raised the level of resistance to pirimiphos-methyl from 3- to 6-fold at LD50. These results may have important implications for the future control of O. surinamensis in farm grain stores.

INTRODUCTION

A recent survey (Taylor & Sly 1986) has shown an increase in the proportion of farms in England using insecticides to control insect pests of stored grain, with pirimiphos-methyl being the insecticide most frequently used. At the same time an increasing proportion of samples of the Saw-toothed Grain Beetle (Oryzaephilus surinamensis) tested for resistance to organophosphorus insecticides at the Slough Laboratory have contained insecticide-resistant individuals. 40% of samples of O. surinamensis collected between August 1984 and July 1986 were resistant to pirimiphos-methyl. Although laboratory experiments (Muggleton 1986) have shown how selection with insecticides increases both the proportion of resistant individuals in a population of O. surinamensis and the resistance factor, we have no information on the effects of insecticide treatment in the field. Nor do we have information on the extent to which recommended treatments will control populations identified as resistant in the laboratory. In an attempt to obtain such information, we have conducted a pilot study on a farm in northern England with a heavy infestation of O. surinamensis resistant to malathion, pirimiphos-methyl, chlorpyrifos methyl, etrimfos, fenitrothion and permethrin.

MATERIALS AND METHODS

The Farm

This is a mixed farm in North Yorkshire with a history of *O. surinamensis* infestation since at least 1972. Wheat and barley produced on the farm are stored in two floor stores. One (the 'old' store) is an old hay drying barn (52 x 8 x 6 metres) with hollow stone walls, an asbestos roof and concrete floor. One side of this barn was originally open and supported by stone pillars, the gaps between the pillars are now filled by corrugated metal sheets. There is a bin and a drier at one end of the store. The second ('new') store (40 x 15 x 5 metres) was built ca. 1982 of asbestos cement panels with a concrete floor. It is contiguous with buildings housing animals and the dividing wall is constructed of breeze blocks. During our investigation, wheat was kept in the new store and barley in the old store. 150 tonnes of barley and 400 tonnes of wheat were stored in 1984, smaller quantities in 1985.

Resistance testing

Adult beetles were exposed to insecticide following FAO Method No. 15 (Anon. 1974), in which beetles are confined on insecticide treated filter papers by glass rings coated with 'Fluon' (an aqueous suspension of polytetrafluorethylene). The insecticides were applied to Whatman No.1 filter papers in a mixture of Risella oil, petroleum ether and acetone in a ratio of 1:3:1. 0.5 ml of this mixture was applied to each filter paper which was then left to dry for 18 hours. Technical grade malathion (95.3% pure), pirimiphos-methyl (85.4% pure) and chlorpyrifos methyl (92.1% pure) were used throughout. The discriminating dose used to detect malathion resistance, 78 mg/m^2, replaced the FAO recommended dose of 260 mg/m^2 (Anon. 1974) which has been found to be too high (J. Muggleton unpublished data). The discriminating dose of pirimiphos-methyl was 156 mg/m^2 (J. Waller and J. Muggleton unpublished data). Adult beetles were kept for at least one week on a culture medium of whole wheat flour, rolled oats and yeast before testing with insecticide. When insufficient numbers were collected for immediate testing the beetles were reared for one generation before testing.

Pirimiphos-methyl dose-response lines for the susceptible strain were obtained using doses between 52 and 104 mg/m^2, plus an untreated control. For the pre- and post-treatment samples doses between 81 and 104 mg/m^2 plus an untreated control were used. Approximately 100 adults were exposed at each dose. Probit regression lines were fitted to the data following the maximum likelihood method of Finney (1971).

PESTICIDE TREATMENTS AND INFESTATIONS

Pre-1985

O. surinamensis was first detected by the farmer in 1972 but no treatments were applied that year. Well established and large infestations were found in the grain and in the fabric of the old store the following yea, and in 1976 and 1978. On each of these occasions the grain was treated with a liquid fumigant and the store sprayed either with malathion (1973 and 1979) or pirimiphos-methyl (1976). On none of these occasions was the treatment complete, some parts of the store and/or the grain remaining untreated. Tests for malathion resistance using a discriminating dose of 260 mg/m^2 malathion in 1976 and 1978 showed samples to be susceptible. There were no treatments prior to harvest in either 1982 or 1984, but in other years spraying of the fabric with malathion and subsequent admixture of the grain with malathion was on an irregular basis.

The old store was inspected in early October 1984, at the request of the farmer; very large numbers of O. surinamensis were moving over the surface of 150 tonnes of barley which was heating to 38°C. Sampling showed that even higher numbers of beetles were present at a depth of 2 metres. A sample of adults was removed and found to be resistant when tested with the 78 mg/m^2 discriminating dose of malathion. In subsequent tests with pirimiphos-methyl these beetles gave 30% knockdown at the discriminating dose. The grain was subsequently treated with phosphine and those parts of the fabric that were accessible were sprayed with pirimiphos-methyl. A post-treatment visit to the farm at the end of October suggested that the treatment had been successful as no beetles were found in the barley or on the fabric. At the same time the 400 tonnes of wheat in the new store was inspected and a smaller, more localised infestation of O. surinamensis was found. These beetles were also found to be resistant to both malathion and pirimiphos-methyl. 240 tonnes of this wheat were sold, and the remaining 160 tonnes were used on the farm as animal feed. The wheat remaining on the farm was admixed with pirimiphos-methyl by the farmer. A further visit in March 1985 revealed the presence of O. surinamensis in the now empty old store as well as in the new store. When tested these beetles showed an increased frequency of individuals resistant to malathion compared to the samples collected in October, but were fully susceptible to phosphine.

1985

The existence of a large infestation of O. surinamensis on this farm, coupled with a high frequency of individuals resistant to malathion and pirimiphos-methyl and a history of ineffective insecticide treatments suggested the site as one where the effects of a fabric treatment at a MAFF recommended dose could be monitored. Live adults were collected by hand from the north, south and west sides of the old store and from wheat residues at one point in the new store. These samples were kept separate and were reared in the laboratory prior to testing with pirimiphos-methyl and chlorpyrifos methyl. Samples of adults were exposed on filter papers treated with 156 mg/m^2 of pirimiphos-methyl or chlorpyrifos methyl (Table 1).

TABLE 1

Percentage of adult O. surinamensis knocked-down when exposed to 156 mg/m^2 of pirimiphos-methyl (pm) or chlorpyrifos methyl (cpm) on filter papers for 5 hours. tests between numbers alive and dead show no significant difference between sites.

Site	% knockdown pm	n	% knockdown cpm	n
Old store:				
North side	50.6	79	85.2	81
South side	51.9	104	75.6	90
West side	48.7	117	81.9	116
New store	48.2	112	71.2	111
χ^2 (3 d.f.)	0.38		6.82	

On 18 June both stores were thoroughly cleaned by MAFF staff and the surfaces treated with 40% pirimiphos-methyl ('Actellic') wettable powder diluted with tap water and applied with a motorised knapsack sprayer to achieve a mean deposit of 400 mg/m^2. The animal house adjacent to the new

store was left untreated. After the treatment, bait bags (Jacobson & Pinniger 1982) were laid in both stores to collect any survivors of the treatment. The bait bags were examined for live and dead O. surinamensis 8, 20, 48 and 57 days after the treatment. Table 2 shows the number of live and dead adults in the bait bags on 20th and 48th days after treatment. The living beetles were removed from the bags, reared in the laboratory and tested for pirimiphos-methyl resistance. The percentage resistant to pirimiphos-methyl are shown in Table 3; the survivors from bait bags have been combined to give one value for each of the three sides of the old store and one value for the new store.

TABLE 2

Numbers and percentage of live and dead adult O. surinamensis found in bait bags placed in the old store 20 and 48 days after treatment

Location and numbers of bait bags	20 days			48 days		
	alive	dead	% alive	alive	dead	% alive
North side (5 bags)	43	131	24.7	19	177	10.7
West side (3 bags)	31	216	12.6	0	390	0
South side (5 bags)	19	122	13.5	3	245	1.2

TABLE 3

Samples of adult beetles from pre- and post-treatment samples knocked-down by the discriminating dose for pirimiphos-methyl resistance. Samples from the treated sites (*) were reared before testing. Numbers tested in parenthesis

Location	Old Store			New Store	Animal house
Sampling date	north side	south side	west side		
30.5.85	50.6(79)	51.9(104)	48.2(112)	48.7(117)	-
8.7.85	15.6(243)*	25.4(110)*	13.6(103)*	23.2(108)*	46.8(248)
14.8.85	13.3(114)*	20.2(124)*	-	22.2(117)*	-

In mid-August, wheat and barley from the 1985 harvest was put into store by the farmer. As before, the barley was placed in the old store and the wheat in the new store. Prior to storage the farmer admixed the grain with pirimiphos-methyl. On 16th October the farm was again visited and probe and pitfall traps (Barak & Harein 1982, Cogan et al In press) were placed in both the wheat and the barley. Visual inspection and sieving samples of grain yielded only a single O. surinamensis in the old store. However moderate numbers of live O. surinamensis were present in the walls and residues in the animal house adjoining the new store. These were collected and reared, and when tested for resistance to pirimiphos-methyl gave 51% knockdown. Analysis of the grain samples showed levels of around 1.4 mg/kg of pirimiphos-methyl in the barley and 0.25 mg/kg in the wheat, this is considerably lower than the target dose of 4 mg/kg.

The traps were inspected for O. surinamensis at fortnightly intervals and the first live beetles were found in both stores on 28 November. Further collections of beetles were made on the 9th and 19th December, during which time the numbers in the old store increased and on 19 December the barley was reported to be heating and the beetles were very active. Very few beetles were found in the traps in the new store. A final visit on 7 January 1986 yielded 430 O. surinamensis from 8 pitfall and 8 probe traps, the majority were from the part of the grain found to be heating on the previous visit and which had now been used up. The temperature of the remaining barley ranged from 8.5 - 9.0°C and the beetles were inactive. Further samples of the grain were removed for insecticide residue analysis and this showed a mean 1.26 mg/kg in the barley and 0.23 mg/kg in the wheat. The beetles collected in the old store in December and January were tested with pirimiphos-methyl (Table 4). There were sufficient beetles at trap sites 4 and 8 to be tested separately. The beetles from the remaining trap sites were combined, although most came from site 7.

TABLE 4

Percentage of adults knocked-down by the discriminating dose for pirimiphos-methyl. The samples were collected from probe and pitfall traps in the grain and were reared before testing for resistance. Numbers tested are in parenthesis.

Trap site	B4	B8	B7 + others
Sampling date			
9.12.85	21.1(145)	23.8(122)	2.4(204)
19.12.85	20.2(114)	26.5(98)	-
7. 1.86	26.7(120)	24.8(121)	12.6(214)

DISCUSSION

Tests on the insects collected on 30 May suggested a uniform frequency of individuals resistant to pirimiphos-methyl, both within the old store and between the old and new stores (Table 1). As a further check on uniformity the beetles from these samples were also tested with chlorpyrifos methyl. Resistance to the two compounds is inherited separately in other strains of O. surinamensis (J. Muggleton, unpublished data). The uniformity in response to both insecticides suggested the existence of one interbreeding population or, less likely, a number of populations subject to the same selection pressure. The beetles in the animal house adjoining the new store also seem to be part of this population as beetles collected there on 8 July (Table 3) showed similar knockdown by pirimiphos-methyl as those from the old and new stores on 30 May (χ^2 = 2.81).

Following the treatment of both stores on 18 June there was a significant increase at each sampling point ($P<0.001$) in the proportion of adults resistant to pirimiphos-methyl compared to samples collected on 30 May. Furthermore, on this occasion there was evidence of heterogeneity between the sites (χ^2 = 8.0, $P<0.05$), the frequency of knockdown being lower on the north and west sides of the old store than elsewhere. The greater part of the north side is of metal construction with few cracks and crevices; however adjacent to the entrance is a grain drying area with many harbourages. If the beetles from this latter area are excluded the frequency of knockdown for the north side falls to 7.4% (n = 121). This difference would

suggest that the selection pressure on the population on the north and west sides of the store had been greater than that on the south side and in the new store. However the area adjacent to the entrance on the north side appears to belong to the latter group as here the knockdown was 23.8% (n = 122). Tables 2 and 3 show that although the frequency of resistant beetles had increased post-treatment, the number of beetles found alive in the bait bags was very small. Again the sampling point adjacent to the entrance on the north side of the old store gave an anomalous result with 86% alive (n = 36). The highest numbers (alive + dead) were found on the west side of the old store. By August there were few live beetles. Beetles collected from bait bags on 14 August (Table 3) showed frequencies of resistance similar to those collected on 8 July but the differences between the sites were not significant (χ^2 = 3.44). A sample collected from the animal house on 5 August gave 57.9% knockdown (n = 240) to pirimiphos-methyl after being bred-up, compared to 42.9% (n = 191) for adults bred-up from the sample collected on 8 July, suggesting a decrease in the untreated area (χ^2 = 9.56, P<0.01). A further sample from the animal house collected on 16 October gave 50.9% knockdown to pirimiphos-methyl after rearing (n = 108) indicating no further change in the frequency of resistance.

The samples from the traps in the barley, collected at the end of 1985 generally showed no change in the frequency of resistance (Table 4) from those collected from bait bags in mid-August. For samples collected at trap sites B4 and B8 there was no significant difference between the numbers knocked-down on 9 December and those collected from the bait bags on 14 August (χ^2 = 2.99). Nor was there any significant change in numbers knocked-down in samples collected at B4 and B8 on 9 and 19 December and 7 January (χ^2 = 2.07). However the knockdown for beetles collected from the other trap sites was significantly lower than at B4 and B8. A chi-squared test on the numbers alive and knocked-down for all samples on 9 December shows significant heterogeneity between the samples (χ^2 = 40.08, P<0.001) and the same was true for those collected on 19 December (χ^2 = 12.51, P<0.01). At the other sites there was a significant increase in knockdown between 9 December and 7 January (χ^2 = 15.27, P<0.001). The overall picture after treatment was of a general increase in the frequency of resistance throughout the treated areas, but with some localised areas showing even greater increases. It is possible that this patchiness in response was due to uneven pesticide application (or by the treatment being more effective on one type of surface than another) and isolation of the surviving populations.

This increase in the frequency of resistant individuals does not necessarily mean that the resistant individuals in the selected populations were able to survive higher doses than resistant individuals present before selection, it only means that the frequency of the resistant gene has increased at the expense of the susceptible gene. To test whether there was an increase in the dose that can be survived requires dose response data for treated and untreated populations. To make this comparison we used beetles collected from the barley on 9 December 1985 (excluding those from sites B4 and B8) to represent the treated population and those collected from the animal house on 16 October 1985 to represent the untreated population. The beetles from the animal house would thus have been in culture for one generation longer than those from the barley. Dose-response lines for these two populations are shown in Figure 1. The resistance factor of the treated population compared with the untreated, was ca. 2 at both LD50 and LD99.9 indicating that the treatment increased the population's ability to survive higher doses of insecticide. If the change had been merely to increase the frequency of the resistance gene we would have expected to see a steepening

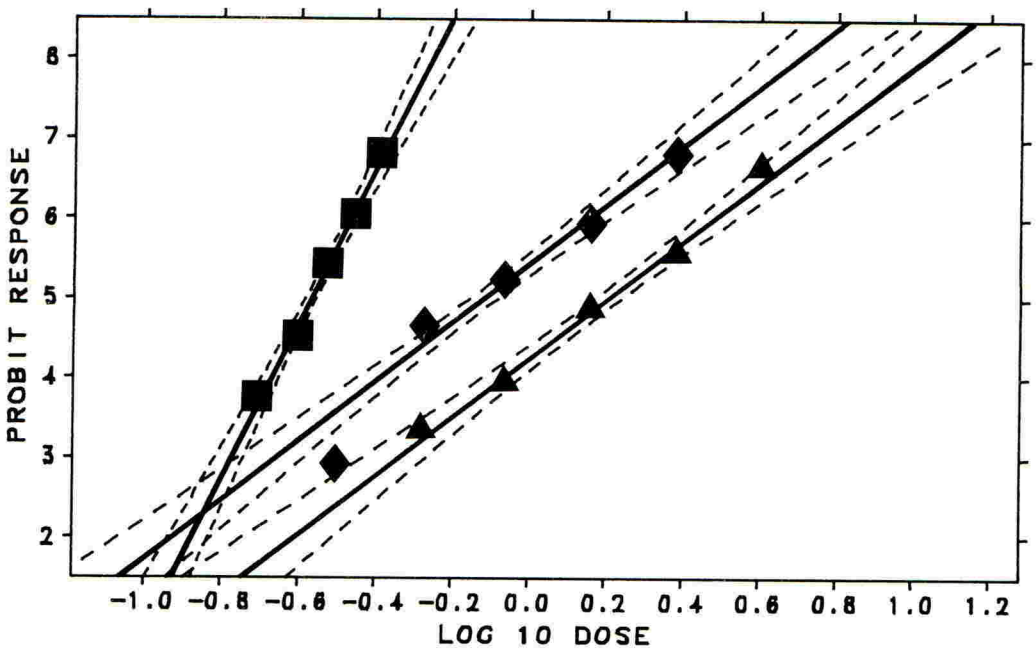

Fig. 1. Dose-response lines for O. surinamensis with 95% Fiducial limits. Key: ■ Laboratory susceptible strain, ◆ beetles from untreated animal house, ▲ beetles collected from old store after treatment.

of the line for the treated population and an increase in the LD50 but not the LD99.9. The untreated population had a resistance factor of approximately 3x that of a susceptible laboratory population.

This 2-fold increase in resistance as a result of insecticide selection in the field was very similar to that obtained with a similar dose of malathion in a laboratory selection experiment (Muggleton 1986), in which the frequency of the malathion resistance gene rose from 0.51 to 0.79 in one generation as a result of selection. The discriminating dose for pirimiphos-methyl is too high to allow a direct estimate of the frequency of the pirimiphos-methyl gene, because it kills a proportion of the beetles heterozygous for resistance. However, if we assume, that as in other strains, malathion and pirimiphos-methyl resistance are controlled by the same gene and that this gene is present in the farm population, we can apply a correction factor to the results of the pirimiphos-methyl discriminating dose tests to give a better estimate of the frequency of the resistance gene. Making this assumption, the change in frequency of the resistance gene between samples collected on 30 May and 14 August was from 0.41 to 0.7. Thus there appears to be general agreement between the laboratory experiments and the field observations.

Finally, it is necessary to consider efficacy of control in the two stores. Although the fabric treatment was applied as accurately and thoroughly as possible, the admixture of insecticide with grain by the farmer was erratic and resulted in underdosing. It is therefore difficult to determine the major factors contributing to insect survival. This emphasises the importance of accurate dosing of insecticide treatments. In both stores

the number of beetles caught after treatment was small. By contrast two bait bags laid in the untreated animal house yielded more than 700 live beetles each. At the final visit, relatively few beetles were collected from a large number of bait bags placed end to end along the north and south sides of the old store. However, it may be significant that on this final visit to collect bait bags, live beetles were still present and the proportion found alive had increased. The number of beetles found in the new store, where the initial infestation had been smaller, was low and subsequently few beetles were found in this store; the new crop of grain remains uninfested. In the old store, beetles were present and infesting the grain by December 1985. However the subsequent removal of the infested part of the grain and a prolonged spell of cold weather may have prevented the infestation from spreading. Fifteen bait bags put in the empty old store and examined on 6, 14 and 20 June 1986 yielded 4, 14 and 20 beetles respectively. So a remnant population of O. surinamensis still exists in the old store and the treatment here cannot be regarded as completely successful although it may be argued that the many harbourages in the old store would have protected a part of the population from pesticide treatment. On the other hand the fact that the proportion of resistant animals increased after treatment indicates that the insecticide had reached and killed susceptible beetles and there is no evidence of a subsequent emergence of protected susceptible beetles from the harbourages.

The increase in incidence of insecticide resistant O. surinamensis on UK farms, coupled with the selection for resistance demonstrated on this farm, implies an increased risk of treatment failure with pirimiphos-methyl and related compounds.

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THE GEOGRAPHICAL SPREAD OF RESISTANCE TO PHOSPHINE BY COLEOPTEROUS PESTS OF STORED PRODUCTS

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ABSTRACT

Phosphine is now the major fumigant used for the disinfestation of stored commodities such as cereal grains. It is particularly employed in tropical and sub-tropical regions where the warm conditions enhance its effectiveness. Unfortunately phosphine fumigations are often carried out under unsatisfactory conditions of sealing in which lethal concentrations of gas are maintained for insufficient periods to ensure 100% or near 100% mortality of all stages of insect development. This has resulted in the development of resistance to phosphine.

Following the detection of resistant strains of insects in food stores in Bangladesh in 1982, TDRI has instituted a survey of the incidence and level of resistance to phosphine in strains of key species of insect pests of stored grains in developing countries throughout the world. An initial screening using a discriminating dose of phosphine was carried out on adult insects. Any strains indicated to be of reduced susceptibility to phosphine were subjected to further examination to determine their capability to survive gas concentrations and exposure times likely to occur in field treatments.

Resistance to phosphine has so far been detected in strains of Rhyzopertha dominica, Tribolium castaneum, Sitophilus oryzae, Oryzaephilus surinamensis and Cryptolestes ferrugineus. These strains were collected in one or more countries including in addition to Bangladesh, Bhutan, Brazil, Mali, Nepal, Pakistan and Sri Lanka.

INTRODUCTION

The disinfestation of food commodities by fumigation is a major means of preventing damage by insect pests during storage, especially in tropical countries. Of the few fumigants available, the most widely used is phosphine which is generated from solid preparations of aluminium or magnesium phosphide, when these are exposed to the moisture naturally present in air.

Phosphine has now been in regular use for more than 20 years for controlling insect pests in both bagged and bulk grain. In 1981 problems of controlling insect pests, especially the lesser grain borer (Rhyzopertha dominica), and the rust-red flour beetle (Tribolium granarium), were experienced in Bangladesh. A survey by a team from Tropical Development and Research Institute confirmed that local strains of insects could survive phosphine treatments which in most instances killed strains of normal tolerance to phosphine (Tyler et al. 1983). Laboratory assessments later confirmed these field strains of insects to be more resistant to phosphine than any previously tested (Mills 1983).

Following this first documentation of fumigation failure in field strains, due to a reduced response to phosphine, an investigational programme was started by TDRI to discover the extent to which resistance to fumigants had developed in other countries, and how this might affect present recommendations for applying phosphine in pest control programmes.

MATERIALS AND METHODS

Insects were obtained from 18 tropical countries, including in addition to R. dominica and T. castaneum, the maize weevil (Sitophilus zeamais), the rice weevil (Sitophilus oryzae), and the saw-toothed grain beetle (Oryzaephilus surinamensis). Where insufficient numbers were initially available for testing, cultures were reared in a CTH room maintained at a temperature of 27°C and a relative humidity of 70%.

All insect strains were first exposed to a discriminating dose of phosphine in desiccators as adult insects, in accordance with the method recommended by FAO (Anon. 1975). Where 100 per cent mortality was obtained in the first screening, strains were assumed to be phosphine-susceptible and no further evaluations were made. Where a high level of survival occurred, further tests were carried out using adult insects at higher concentrations and with longer periods of exposure. Variation of both concentration and exposure period was necessary because of the ability of insects to limit initially rate of uptake of phosphine by entering a state of depressed metabolism, often described as narcosis (Bell 1986).

RESULTS AND DISCUSSION

The results of insect mortalities at the discriminating concentrations and at increased concentrations of phosphine and longer exposure periods, are shown in Tables 1 - 3.

At the discriminating dose level there was a wide range of response by insects tested, varying from nil to 100 per cent. In countries such as Pakistan and Mali from where a large number of strains have been examined, this range of reponse is also recorded within the country. It is from these two countries that the greatest reduction in susceptibility to phosphine has been recorded in the present programme.

A strain of T. castaneum from Qasim (Pakistan), showed a nil mortality at 0.3 mg/l phosphine for a 48-hour exposure period, and a strain of R. dominica from Severe (Mali), showed only 7% mortality at 0.4 mg/l phosphine during a 72-hour exposure period. Insects collected from Mopti in Mali in 1983 demonstrated an even lower response to phosphine; only 2% of a strain of R. dominica died when exposed to 0.3 mg/l for 6 days. Survival of adult insects at this order of fumigant concentration and exposure period, which are what might be expected in well conducted fumigations, suggests that the required level of insect control would not be achieved under practical conditions.

Poor response of insect pests to phosphine can in some circumstances be associated with situations where there has been a history of poor fumigation techniques being employed. Inadequacies in technique include poor sealing leading to rapid loss of gas and inadequate exposure periods. Such practices are common in the Indian Sub-Continent where the fumigation of whole stores is commonly carried out under conditions of poor sealing. Such fumigations control the more phosphine-susceptible members of the insect population but allow the more tolerant members to survive and breed, leading to the

eventual dominance of strains which are resistant to phosphine. This type of situation also occurs in Brazil where frequent fumigations of uncovered bulk grain are carried out and where species resistance to phosphine have been identified.

The effect of poor quality treatments on the development of phosphine resistance is well demonstrated in strains of T. castaneum from Karachi. Strains 1 and 2 which showed a nil response to the discriminating dose were collected from food stores where frequent treatments under non gas-tight conditions are carried out and where, therefore, selection is likely to occur. Strains 3, 4 and 5 of T. castaneum were collected from feed mills in Karachi where, because little or no fumigation is practiced, there is correspondingly little selection pressure. In consequence these strains showed 88%, 100% and 81% response to discriminating dose tests and can still be regarded as relatively susceptible.

There is evidence to suggest in some instances where insects have been found to be resistant to phosphine that this did not develop locally and that these strains were imported from elsewhere, usually outside the country, on infested commodities. Insects collected in Bhutan, for example, where virtually no phosphine fumigation has occurred in the past, were found to be resistant to phosphine. The infested commodities containing the insects had been imported from, or through India, indicating that resistance may have developed in that country. A similar situation may have been the cause of resistant insects being found in Nepal which borders India and through which most, if not all, food imports pass. Strains of R. dominica collected in Sri Lanka and found to be resistant to phosphine were from rice recently imported from Pakistan and may therefore have travelled on rice from that country.

It seems likely that the transfer of phosphine-resistant strains on infested commodities from one country to another is now not uncommon. This has important implications for phytosanitary inspection procedures by importing countries, which should not be ignored. A strain of Cryptolestes ferrugineus described as highly resistant to phosphine has already been intercepted in Britain on commodities imported from India, and another strain was collected from a provender mill in England, again almost certainly imported from a country where fumigation practice is below acceptable standards (Dyte and Halliday 1985).

The degree of overkill built into manufacturers' recommendations for phosphine fumigation was in the past probably sufficient to give adequate or near adequate control of insects even in poorly conducted treatments. Selection of insect populations less responsive to phosphine has resulted in much less than adequate control now being achieved in poorly conducted fumigations. Satisfactory control of these populations is, however, likely to occur in properly conducted treatments in well-sealed enclosures.

There is every indication that strains of insect pests of stored products that are no longer adequately controlled in poor, or even mediocre quality treatments, are now widely dispersed in some regions. If the standard of fumigation could be raised in these areas, there is every chance that resistance by insect pests to phosphine might not become a serious problem. However, because of the very few alternative fumigants available for disinfestation of food commodities, the loss of effectiveness of phosphine as a major means of insect control in stored foodstuffs would be a serious blow to those responsible for storing them, especially in developing countries.

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TABLE 1.

Response to phosphine of insect strains collected from the Indian sub-continent

Location collected	Year	Species	% Mortality at discriminating concentration*	Increased concentration (mg/l)	Exposure period (hours)	% Mortality
BHUTAN						
Phuntsholing	1985	T.castaneum	1	0.1	48	28
				0.1	5(days)	70
Phuntsholing	1985	S.oryzae	100	NT		
NEPAL						
Biratnagar	1984	T.castaneum	22	0.1	48	85
Nepalgunj	1984	S.oryzae	82	NT		
Janakpur	1984	R.dominica	2	0.3	6(days)	83
				0.6	6(days)	100
PAKISTAN						
Karachi	1984	R.dominica	12	NT		
Karachi 1	1985	T.castaneum	0	0.3	7(days)	99
Karachi 2	1985	T.castaneum	0	0.04	7(days)	0
Karachi 3	1985	T.castaneum	88	NT		
Karachi 4	1985	T.castaneum	100	NT		
Karachi 5	1985	T.castaneum	81	NT		
Karachi	1985	S.oryzae	100	NT		
Okara	1985	T.castaneum	0	0.04	7(days)	0
Peshawar	1984	R.dominica	8	0.1	72	73
				0.4	72	96
Qasim	1985	T.castaneum	0	0.3	48	0
SRI LANKA						
Colombo 1	1984	R.dominica	100	NT		
Colombo 2	1984	R.dominica	52	0.1	20	88
				0.2	20	99
Colombo 1	1986	R.dominica	1	0.1	72	7
				0.2	72	7
				0.4	72	49
Colombo 2	1986	R.dominica	3	NT		
Colombo	1986	O.surinamensis	5	NT		
Colombo	1986	T.castaneum	85	NT		

* R. dominica 0.03 mg/l 20 hours exposure
T. castaneum 0.04 mg/l 20 hours exposure
Sitophilus spp. 0.04 mg/l 20 hours exposure
O. surinamensis 0.05 mg/l 20 hours exposure

NT = Not tested.

TABLE 2.

Response to phosphine of insect strains collected in Africa

Location collected	Year	Species	% Mortality at discriminating concentration*	Increased concentration (mg/l)	Exposure period (hours)	% Mortality
BOTSWANA						
Pitsane	1985	R.dominica	4	0.10	20	94
ETHIOPIA						
Assab	1985	T.castaneum	100	NT		
GHANA						
Kumasi	1986	S.zeamais	100	NT		
LIBERIA						
Gbarnga	1985	T.castaneum	100	NT		
MALI						
Mopti	1983	R.dominica	0	0.30	5(days)	2
				0.65	3(days)	13
				0.65	6(days)	100
Bamako	1985	R.dominica	50	NT		
Bamako	1985	T.castaneum	49	NT		
Kati	1985	T.castaneum	100	NT		
Kayes	1985	R.dominica	39	NT		
Koulikoro	1985	R.dominica	91	NT		
Niafunke	1986	T.castaneum	0	0.1	72	0
				0.4	72	100
Segou	1985	R.dominica	87	NT		
Severe 1	1985	R.dominica	3	NT		
Severe 2	1985	R.dominica	100	NT		
Severe 3	1986	R.dominica	0	0.1	72	0
				0.4	72	34
Sikasso	1985	R.dominica	86	NT		
NIGERIA						
Maidugari	1985	R.dominica	100	NT		
TUNISIA						
Florence	1985	S.oryzae	88	NT		
Kairouah	1985	R.dominica	93	NT		
Sousse	1985	T.confusum	100	NT		
ZIMBABWE						
Causeway	1985	S.zeamais	100	NT		
Mutare	1985	R.dominica	100	NT		
Mutare	1985	T.castaneum	100	NT		

* R. dominica 0.03mg/l 20 hours exposure
T. castaneum 0.04mg/l 20 hours exposure
Sitophilus spp. 0.04mg/l 20 hours exposure

NT = Not tested.

TABLE 3.

Response to phosphine of insect strains collected in other areas

Location collected	Year	Species	% Mortality at Discriminating Concentration*	Increased Concentration mg/l	Exposure period (hours)	% Mortality
BRAZIL						
Assis	1986	S.oryzae	9	0.1	24	43
				0.1	72	99
				0.4	72	100
	1986	O.surinamensis	35	NT		
	1986	T. castaneum	5	0.1	48	99
Ourinhos	1986	S.oryzae	15	NT		
	1986	T. castaneum	0	0.1	72	66
				0.4	72	100
Sumare	1986	S.oryzae	6	NT		
Tatui	1986	S.oryzae	48	NT		
BURMA						
Mandalay	1985	T.castaneum	100	NT		
	1985	S.zeamais	100	NT		
INDONESIA						
Jakarta 1	1986	T.castaneum	100	NT		
Jakarta 2	1986	T.castaneum	100	NT		
Jakarta 3	1986	T.castaneum	100	NT		
Jakarta 4	1986	T.castaneum	100	NT		
Jakarta 5	1986	T.castaneum	100	NT		
Jakarta 6	1986	T.castaneum	100	NT		
Jakarta 7	1986	T.castaneum	100	NT		
Njarjuk	1986	R.dominica	0	0.1	72	0
				0.4	72	26
SINGAPORE (Ex North America)	1985	R.dominica	100	NT		
ST LUCIA						
Castries	1985	T. castaneum	100	NT		
THAILAND						
Lopburi	1985	S.zeamais	99	NT		

* R. dominica 0.03 mg/l 20 hours exposure
T. castaneum 0.04 mg/l 20 hours exposure
Sitophilus spp. 0.04 mg/l 20 hours exposure
O. surinamensis 0.05 mg/l 20 hours exposure

NT = Not tested.

POTENTIAL HOUSEFLY CONTROL IN INTENSIVE ANIMAL UNITS USING ENTOMOPHILIC NEMATODES

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ABSTRACT

Immature stages of an insecticide-resistant field strain of Musca domestica were exposed on filter papers in the laboratory to infective stages of two species of entomophilic nematodes : Heterorhabditis heliothidis and Steinernema feltiae. All three larval instars were susceptible to the parasites; S. feltiae was the most pathogenic and killed 90% of all larval stages at the highest innoculum levels. No pupae were parasitised by either species. Adult houseflies were exposed to bait pads innoculated with either H. heliothidis, S. feltiae or S. bibionis. Heterorhabditis heliothidis proved to be the most pathogenic species and killed up to 53% of adult houseflies. This control method used alone or to supplement conventional insecticide treatments could substantially reduce the selection pressure for insecticide resistance in this pest.

INTRODUCTION

The housefly (Musca domestica) is frequently found infesting animal units, such as piggeries and poultry houses. The presence of such infestations can cause staff unrest which may result in poor animal management. Houses bordering on farmland can also be affected by housefly migration, resulting in the threat of farm closure by local health authorities. Campbell et al. (1984) observed a reduction in pig weight gain when they introduced a simultaneous infestation of houseflies and stableflies (Stomoxys calcitrans). Loss of profit can also be incurred by eggs rendered unsaleable through egg spotting.

Probably the most significant aspect of housefly infestations is the possible transmission of disease. Gough & Jorgenson (1983) detected porcine transmissible gastroenteritis (TGE) virus in M. domestica collected from a contaminated pig farm. Houseflies have also been implicated in the transmission of Staphylococcus sp from pigs (Hajek & Balusek 1985) together with eggs of pig helminths, such as Taenia solium (Eucestoda) and Trichuris trichuria (Nematoda) which are also capable of parasitizing man (Harwood & James 1979). Greenberg et al. (1963) found that houseflies collected from a slaughter house were infected with Salmonella spp. Harwood & James (1979) stated that the housefly may be able to transmit fowl tapeworm (Choanotaenia infundibulum) or act as a vector for virulent spores of Anthrax (Bascillus anthracis) (Leclercq 1969).

Traditionally housefly control has been achieved with chemicals, but the development of resistance to insecticides has long been a problem. Green (1955) found houseflies resistant to DDT, Gamma-HCH and cyclodiene insecticides. Subsequently more than 20 years of satisfactory control was achieved in the United Kingdom with natural pyrethrins and organophosphorus compounds. Then in 1977 the Regional Pest Service of the Ministry of Agriculture, Fisheries and Food, reported control failures with organophosphorus compounds. Sawicki et al. (1979) found houseflies resistant to natural pyrethrins and synthetic pyrethroids, after a treatment with natural pyrethrins failed to achieve control. Since then resistance to a broad range of insecticides has been recorded (Chapman & Lloyd 1981, Chapman 1984, 1985).

There is therefore a need to develop new methods of housefly control which do not select for insecticide resistance. In recent years, biological control organisms have been used with great success, in a number of pest management programs (Kaya 1985, Richardson 1983). One such group of organisms are the entomophilic nematodes of the Steinernematidae and Heterorhabditidae with their associated pathogenic bacterium (Xenorhabdus spp.), which are capable of parasitising a large variety of insect hosts (Poinar 1979). Bedding (1981) reported low-cost in vitro mass rearing methods for some entomophilic nematode species, which has made the field treatment of some important pests more practical (Richardson 1983).

This paper examines the potential of steinernematid and heterorhabditid nematodes to control both larval and adult houseflies.

MATERIALS AND METHODS

Nematode and housefly strains

Experiments were performed using infective nematode larvae of the following species: Heterorhabditis heliothidis (NZ strain), Steinernema feltiae (Agriotos strain) and Steinernema bibionis (T319 strain), all of which were cultured in vivo in late instar Galleria mellonella larvae. Infective nematodes were extracted from G. mellonella using the Baermann funnel technique and were counted using a Fenwick multichamber counting slide (Doncaster et al. 1967). Musca domestica larvae and adults of a field strain (Sparshott) were used as hosts. These were shown to be significantly resistant to both organophosphorous and synthetic pyrethroid insecticide compounds (T. Nicholas, unpublished data).

Susceptibility of larvae and pupae to parasitism

Housefly pupae and larvae of 1st, 2nd and 3rd instars were exposed to infective nematodes using Kaya & Hara's (1980) modification of the Dutky et al. (1964) technique. Twenty-five insects were each placed in covered 5cm (diameter) x 2.5cm (deep) transparent plastic containers with filter paper lining. Inocula of 50 000, 25 000 or 5 000 infective nematodes, of either H. heliothidis or S. feltiae were then added to the filter paper in 0.5 ml tap water. Each of the treatments, together with the controls, were replicated 8 times. Tests were carried out in the dark at 25°C. Parasitism was assessed by dissections at 24 h (1st instar larvae), 48 h (2nd and 3rd instar larvae) or by allowing adults to emerge from pupae. Further details of this technique have been reported by Renn et al.(1985).

Susceptibility of adult houseflies to parasitism

An initial investigation (Renn et al. 1985) did not allow for free air movement or an alternative food source, so the following modification was carried out. Aluminium cages (approximately 30 x 30 x 30 cm) with two open sides covered in plastic gauze (540 mesh) were used as test arenas. Access to the cage interior was gained via a 12 cm diameter porthole, covered with a stocking sleeve, the open end of which was secured with a spring-steel clip. In each cage an alternative food source was provided in the form of a disposable beaker (25 ml) containing cotton wool soaked in aqueous sugar solution (10% w/v).

Bait pads consisted of two 9 cm discs of capillary matting (each 7 mm in depth). One disc was placed in a petri dish base (9 cm) and in order to provide a moisture source to retard desiccation of the nematodes, 30 ml tap water was added. Wheatfeed, which had previously been found to be the most attractive component of the larval diet (N. Renn, unpublished data), was used as the food attractant. A 10g portion of wheatfeed/tap water mix (2:5) was formed into an annulus around the edge of the second disc of capillary matting. This was placed within the petri-dish base, on the 1st disc. Known numbers of infective nematodes were introduced in 10 ml tap water.

One bait pad was placed centrally on the floor of each cage, and approximately 100 houseflies (50♂and 50♀) were introduced. There were six replicate cages for each treatment and six cages containing bait pads without nematodes were used as controls. Tests were carried out at 25°C and 90% r.h., with a 14h light regime of 22 lux intensity.

After the introduction of the bait pad (time 0), mortality was assessed at 24 h intervals. Dead flies were removed from each cage, and after sexing they were dissected to locate parasitizing nematodes. Experiments were terminated after 6 days, when the surviving flies were anaesthetised with CO_2, sexed and dissected. Nematodes surviving on the bait pad were counted, after extraction for 24 h, using the technique of Whitehead & Hemming (1965).

RESULTS

Susceptibility of larvae and pupae to parasitism

S. feltiae was more pathogenic than H. heliothidis, causing 90% mortality of all larvae at the two highest concentrations (Fig. 1). Of the larvae which had died by 48h, S. feltiae caused septicaemia to occur in 71% of 2nd and 3rd instars, compared with 54% caused by H. heliothidis. The high control mortality of 2nd instar larvae was thought to be due to food deprivation. Pupae were not susceptible to parasitism by either species. Further details are given by Renn et al. (1985).

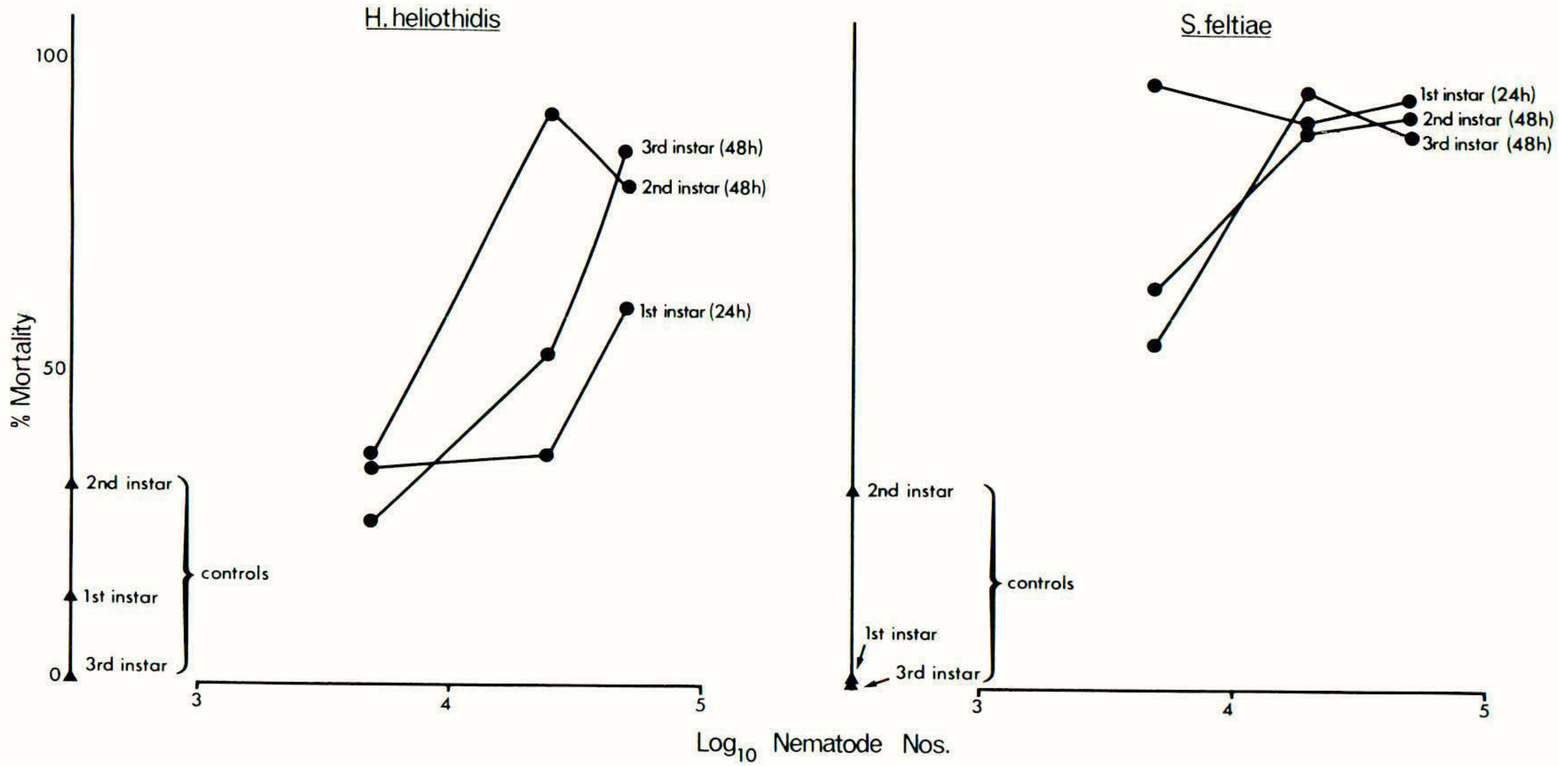

Fig 1. Mortality of M. domestica larvae after exposure to nematodes on moist filter paper. Points are plotted as combined replicate means. Control mortality is shown as the response to zero nematodes. Exposure times are shown in brackets after the larval stages. First instars were exposed for 24h only, since control mortality was high after 48h.

Susceptibility of adult houseflies to parasitism

TABLE 1

Numbers of adult female houseflies dying after exposure to three different nematode species (1×10^6/pad; 8 replicates of 50 flies)

	Day 3 mean ± S.E.	Day 6 mean ± S.E.
H. heliothidis	12.5 ± 2.7	26.8 ± 1.9
S. bibionis	8.5 ± 1.5	19.0 ± 2.6
S. feltiae	9.3 ± 1.6	17.3 ± 2.3
Controls	1.0 ± 0.5	3.8 ± 1.4

When adult houseflies were exposed to bait pads containing infective nematodes, no significant difference ($\chi^2 = 2.12$, $P > 0.05$) was observed between numbers of female houseflies killed by each species by the third day (Table 1). At day 6 H. heliothidis had killed significantly more adult female houseflies than either S. feltiae ($\chi^2 = 5.61$, $P<0.05$) or S. bibionis ($\chi^2 = 4.46$, $P <0.05$) (Table 1). However, the numbers of adult female houseflies killed by S. feltiae or S. bibionis were not significantly different ($\chi^2 = 0.33$, $P >0.05$). Significantly less male than female flies were killed by all three nematode species ($\chi^2 = 4.55$, $P <0.05$).

Dissections of adult houseflies revealed nematodes most frequently in the thorax and abdomen and only infrequently in the head. No differences in the distribution of the different nematode species throughout the moribund fly tissues was observed. None of the flies surviving after 6 days were parasitized.

The proportion of nematodes surviving on the pads after 6 days was variable. The pad extracts showed that between 0 and 50% of nematodes could survive within replicates of individual tests. Observations indicated that the depletion of nematodes was due to the drying of the pad, causing death through desiccation. Migration of the nematodes from the pad surface may have been another factor.

DISCUSSION

When applied to filter paper, both S. feltiae and H. heliothidis at the two higher innoculum levels were able to parasitize and kill housefly larvae of all ages. At all concentrations, S. feltiae proved to be the most pathogenic of the two species. The ineffectiveness of H. heliothidis at the lowest concentration agrees with the findings of Khan et al. (1976). These workers found that M. domestica were not susceptible to parasitism by 3000 H. heliothidis placed in petri-dishes.

The lack of parasitism observed in pupae conflicts with Pionar (1979), who stated that M. domestica pupae were susceptible to parasitism by S. feltiae. However, for S. feltiae and perhaps H. heliothidis the only entry portal to intact pupae of M. domestica is via the spiracles, but the presence of spiracular slits within these openings may have prevented penetration (Bedding & Molyneux 1983).

When nematodes were incorporated into bait pads H. heliothidis proved to be the most pathogenic species to adult houseflies. However, previous data (Renn et al. 1985) suggested that S. feltiae was more pathogenic than H. heliothidis. In addition, during the current experiments, fewer flies were killed than in the previous tests. The differences achieved probably reflect the modification of the technique. The additional food source used here, absent during the initial tests (Renn et al. 1985), was probably an important factor. Initially the arenas used by Renn et al. (1985) were of a smaller capacity (6.5 litres) than those used here (27 litres), so the alternative fly densities may have caused the differing results. Lastly, the fly sex pheromone Z-9tricosene ('Muscamone'), which was used by Renn et al. (1985) perhaps made the bait pads more attractive and thus caused a higher fly mortality. The effects of Z-9tricosene will need to be investigated in subsequent tests.

Infective juvenile nematodes are attracted to insects (Bedding & Akhurst 1975) and invade the host via the mouth, anus or spiracles (Poinar 1979). In addition H. heliothidis infective juveniles are able to penetrate via more membranous parts of the cuticle (Richardson & Hughes 1984). From these data there does not appear to be any preferential differences in the way nematodes enter adult M. domestica. Nematodes may enter adult flies via the thoracic spiracles or the cloaca and as more female than male flies succumbed to parasitism, it is possible that their increased susceptibility may have been a function of ovipositioning behaviour.

During these experiments a high degree of variability was observed, not only between individual tests, but also between individual treatment replicates of the same test. This may have been caused by the similar degree of variability in the numbers of nematodes extracted from the bait pads at the end of the test period. In future experiments therefore, the survival time of nematodes on the pads needs to be prolonged by changing presentation or food media. These experiments were carried-out at a high humidity and anti-desiccants are needed for experiments carried out at the lower humidities (70%) found on pig and poultry farms. Kaya (1985) stated that anti-desiccants improve the survival of S. feltiae but knowledge of formulating nematodes is still limited.

The techniques described here give the frame-work of a promising method to supplement insecticide treatments and which will reduce selection pressure for insecticide resistance and thus allow for continued successful housefly control. Rutherford et al. (1984) have indicated that the three nematodes species investigated here are able to reduce housefly emergence when applied directly to chicken manure surface (see also Pringle 1985). It would also seem likely that emerging adults would also be highly susceptible to nematodes applied in this way. These aspects need to be investigated by means of small-scale field trials, using S. feltiae, S. bibionis and H. heliothidis applied directly to chicken and pig manure.

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EFFECT OF DIFFERENT CONTROL STRATEGIES ON THE DEVELOPMENT OF INSECTICIDE RESISTANCE BY HOUSEFLIES: EXPERIENCE FROM DANISH FARMS.

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ABSTRACT

Trials of different control strategies against houseflies (Musca domestica L.) on Danish farms and monitoring of resistance levels between 1948 and 1985 have shown that residual spraying of fly resting sites is an efficient way of selecting for insecticide resistance. Alternative methods of fly control aimed at reducing the selection pressure have been tried and some widely used. These include (1) restricting residual spraying to 30 or 10% of sprayable surfaces, (2) use of impregnated ribbons suspended under the ceiling, (3) use of toxic sugar baits painted on strategic places, (4) use of non-residual space sprays and (5) combining moderate use of a toxic bait and unrelated larvicide. The results show that strategies (2)-(5) may be useful for reducing problems due to development of resistance, but trials with strategy (1) were not successful.

INTRODUCTION

In areas of pest control where pesticide resistance is a problem it is very important to develop and use control strategies that reduce the selection for resistance. As this is the main theme of the present session we think it is relevant to review briefly experience gained from trials and the practical control of houseflies (Musca domestica L.) on Danish farms spanning almost four decades.

The development of resistance by Danish houseflies to a variety of insecticides 1945-1976 was reviewed and discussed by Keiding (1977). Later developments have been recorded in the Annual Reports of the Danish Pest Infestation Laboratory (DPIL). The history of insecticide usage and resistance problems in Denmark is summarized in Fig. 1. In this paper we focus on the relationship between various methods and strategies that have been tested and used in practice for fly control on Danish farms, and the subsequent development of resistance. In the historical review "we" refers to the staff at DPIL concerned with fly control and resistance at the particular time.

MATERIALS AND METHODS

Farms and fly populations

Mainly farms with traditional pig-rearing and/or dairy units have been used in the investigations. Most of our trials on fly control and resistance have been carried out on small and medium-sized farms in North Zealand, the so-called "trial farms". These trials, however, have been supplemented by investigating cases of control failure and by surveys of resistance on farms elsewhere in Denmark. The results have been related to the available information on use and efficacy of insecticides for fly control.

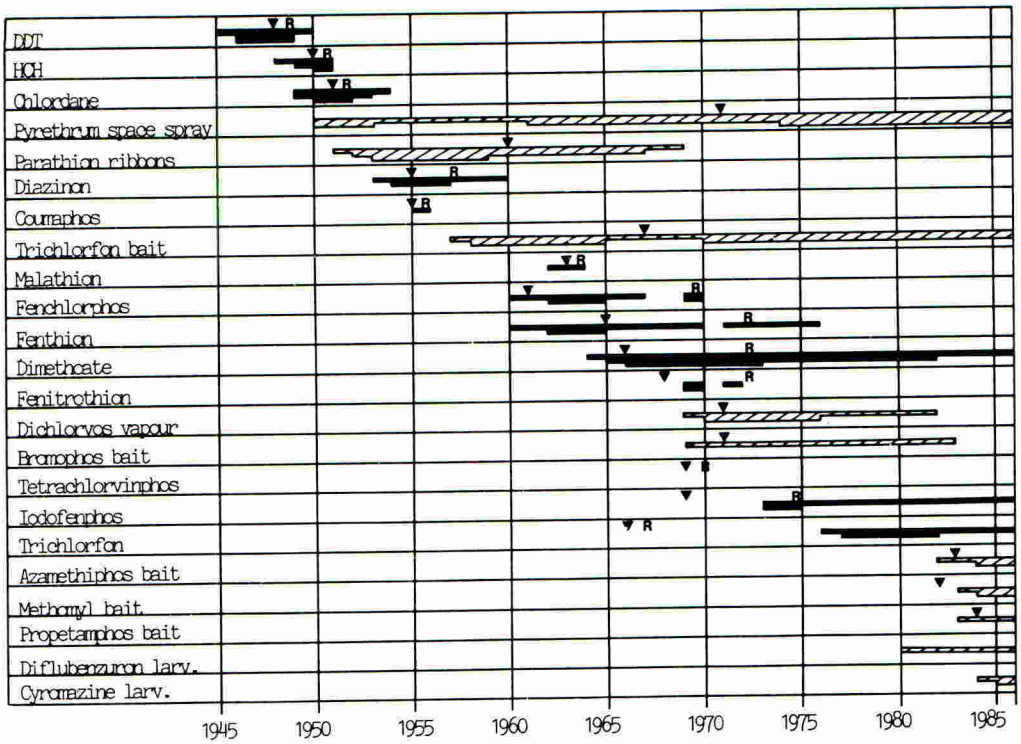

Fig. 1. Countrywide use of insecticides for housefly control on Danish farms 1945-1985 and history of development of resistance. The insecticides were used as residual sprays (shaded areas) except where other applications are stated (hatched areas) Larv.= larvicide. The width of each shaded or hatched area reflects the extent of insecticide usage, on few ═ , many ▭ or most ▭ farms.
▼ = first confirmed case(s) of resistance of practical importance
R = resistance causing general control failure of the method.

(After Keiding 1977, 1986, updated).

Breeding of houseflies, Musca domestica, takes place in pig and calf dung in the animal houses and in summer also in dung heaps outdoors. The "fly season", when control is required, is usually (May)-June-September-(October), depending on the weather. There are about 10-12 generations per year of which 6-8 occur during the fly season. This investigation do not include heated units with longer fly season and more generations. Adult houseflies mostly occur indoors in the animal houses and other rooms at the farm; only on warm days does part of the fly population emerge outdoors around the farm buildings. The flies always spend the night indoors.

Most farms are separated from others by a distance of few to several hundred metres. Their fly populations are generally regarded as relatively self-contained with very restricted movement between farms, either by active migration or transport by cars, trucks, etc. However, if a fly population on a farm becomes very small or zero e.g. due to effective fly control or low temperatures in the animal units, a small contribution of flies from other farms may greatly influence the resistance status of the fly population and thus the interpretation of resistance development over longer periods (Gibson 1981, Denholm et al. 1985). Thus our conclusions on the effect of control strategies on development of resistance are mostly based on the widespread use of a particular method, or on trials where significant genetic contamination or replacement of fly populations from farms with other treatments and resistance levels is unlikely.

On most trial farms some overwintering of houseflies occurs, mainly by slow breeding in calf pens and/or pig units if the temperatures permit, but in unheated piggeries the fly population may die out. In recent years many houseflies overwinter in Denmark by breeding in battery-style poultry units.

Resistance monitoring

On trial farms a representative sample of flies was collected early in the season before the fly control treatments started and once or more later in the season. In surveys of resistance, flies were usually collected only once each year on each farm. When possible, resistance was determined in F_1 progeny of collected flies and expressed as resistance ratios (R/S), relative to a standard susceptible strain at LC_{50}-LC_{95}, and/or by survival at discriminating dosages. Resistance to the contact effect was determined by standard topical application tests. In recent years resistance to the stomach effect has also been determined by feeding male flies sugar containing serial concentrations of the insecticide, and to the larvicidal effect by infesting treated fly medium with newly emerged larvae.

DEVELOPMENT OF RESISTANCE IN RELATION TO DIFFERENT CONTROL METHODS

Throughout this section readers are referred to Fig. 1 and previous reviews (see Keiding 1977).

Residual spraying

As long as the fly population is reasonably susceptible to the insecticide, application of residual spray to all fly resting sites in the animal units is by far the most effective and convenient means of controlling flies on traditional Danish farms. We soon learned, however, that this is also a very efficient way of developing resistance to the insecticide and perhaps other insecticides, depending on the genetic potential for this resistance. Starting from scratch with DDT and probably a very low frequency of a recessive R-gene (i.e. kdr), it took 2-3 years (about 20-30 generations) before control failed due to development of resistance. With some organophosphorus compounds, e.g. coumaphos 1955 and tetrachlorvinphos 1969, high resistance developed in a few months, but in others e.g. dimethoate where resistance was dependent on more factors, (Sawicki, 1975, Sawicki & Keiding, 1981) resistance developed much more slowly.

At an early stage we considered the possibilities of reducing the selection pressure by residual sprays in order to delay the development of resistance. In the early 1960's quantitative studies of the activity,

resting habits and distribution of houseflies in pig and dairy units by day and by night were carried out (Keiding 1965 a). Based on these results (e.g. Fig. 2) we defined those sprayable areas which were most preferred by the flies. For fly control experiments 1966-67 we chose three types of selective spray treatments with dimethoate (1 g per sprayed m^2) covering 10%, 30% and 100% of the sprayable area. Satisfactory fly control was obtained on most farms with 10% coverage, on all farms with 30% coverage and more complete with 100% coverage. However, in 1966 initial resistance to dimethoate of 3-5 fold increased by a factor of 2-4 during the season with 10% and 30% coverage and in 1967 remained high or even increased to higher levels than with the 100% coverage. So the selective spray treatments may save insecticide, but in these trials they were not effective in reducing development of resistance (Keiding & ben Hannine 1972).

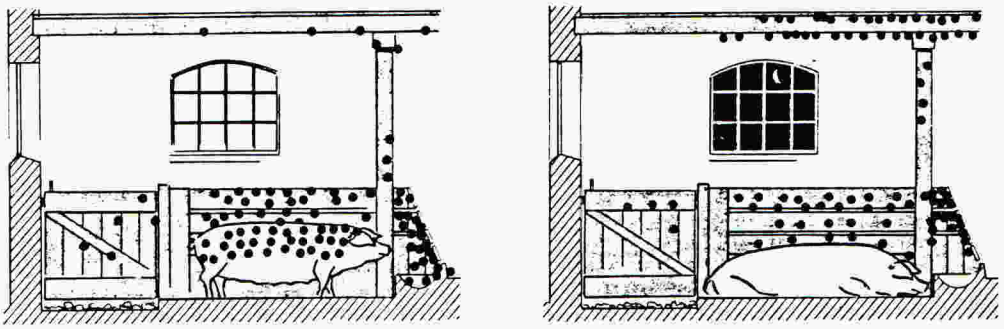

Fig. 2 Vertical distribution of houseflies in a pigsty in a Danish piggery by day (left) and by night. Each dot represents 1% of the fly population. The diagram is based on average counts over several summer days. (After Keiding 1970).

Impregnated ribbons

When residual sprays with chlorinated hydrocarbons failed to control flies in Denmark in the early 1950's, Wichmand (1953) at DPIL got the idea that gauze ribbons impregnated with parathion and suspended under the ceiling in animal units might be used for fly control. Initial trials were encouraging and between 1953 and 65 ribbons impregnated with parathion (2.8 g per m^2 gauze) were widely used for fly control on Danish farms, being the predominant method from 1953 to 58. If the ribbons were correctly installed, preferably early in the season, they could give satisfactory fly control and flies remained fairly susceptible to parathion for several years, e.g. with resistance ratios 2-3 after 4 years' use of parathion ribbons. In subsequent years only a very moderate increase in resistance to parathion was found where the ribbons were used. This contrasted with the development of resistance to other OP-compounds, e.g. coumaphos, diazinon and fenchlorphos, when used as residual sprays (Fig. 1 and Keiding 1965 b, 1977).

Thus it appeared that restricting and concentrating the insecticide to suspended ribbons gave a lower selection pressure for resistance presumably because sufficient flies avoided the contact necessary to pick up a selective dose of parathion. When the fly ribbon method eventually was abandoned it was not because of resistance to parathion. Failing effect was ascribed to the fact that fewer flies contacted the ribbons, due to more ventilation in the animal units and perhaps selection for a change in fly behaviour. Moreover, new and initially more effective residual sprays replaced the ribbons.

Paint-on baits

Fly control with paint-on sugar baits was developed and used in Denmark from 1957, mainly using trichlorphon as the insecticide. Sugar formulations containing 4-10% insecticide either diluted with water (or milk) or ready to use are applied with a paint brush in stripes on strategic places, e.g. on partitions or posts or on suspended boards, strips, etc. Flies are killed when they land and feed on the bait, which may be effective for several weeks. Trichlorphon baits have been widely used on Danish farms since 1958, either as the main fly control method or as a supplement to other methods. It is difficult to obtain effective fly control where the fly breeding potential is high.

The trichlorphon baits caused little or no resistance even after 6-8 years of widespread use. Only occasionally were a few highly resistant flies recorded by the topical tests. Apparently the bait method exerted insufficient selection pressure. However, heterogeneous high resistance developed rapidly after small-scale farm treatments with residual sprays containing trichlorphon (1966-1967), fenthion (1960-1962), dimethoate or bromophos-ethyl (1966). Finally the general field use of dimethoate in the late 1960's resulted in high trichlorphon resistance to the contact effect becoming ubiquitous on Danish farms (Keiding 1977). Trichlorphon bait paint is, nevertheless, still widely used and still kills many flies. Both moderate and high resistance to the stomach effect is found on the farms.

In recent years two other OP-compounds, azamethiphos and propetamphos have been tested and introduced as paint-on baits for fly control on Danish farms. At our advice they were not sold as residual sprays due to the occurrence or risk of rapid development of high resistance to the contact effect, as demonstrated in azamethiphos trials 1981 (Keiding 1982). Before azamethiphos was used resistance to the stomach effect of this compound was moderate in most cases, but increased to heterogenous partly high levels on some farms treated for a season with the bait (Keiding, Skovmand 1984). Current trials with combined use of azamethiphos bait and an unrelated larvicide, with the aim of reducing the selection pressure, are mentioned later in this paper.

Boards prepared with sugar granules containing 1% methomyl (and some fly pheromone) have been tested and used on many Danish farms in recent years. When hung in strategic places they can give a satisfactory fly control without problems of resistance as yet.

Space sprays with non-residual pyrethroids

Space sprays and aerosols containing pyrethrum + piperonyl butoxide (PY-PB) were widely used on Danish farms 1950-70 without serious problems of resistance to PY-PB. For many years these sprays were used to supplement other control methods, but from about 1970 they were increasingly used as

the main fly control method. Trials in 1971 showed that spraying twice a week with PY-PB in the fly season was necessary to obtain satisfactory fly control where the fly breeding potential was high, but such frequent application might induce moderate to high resistance to PY-PB and other pyrethroids. This development of resistance to pyrethroids by frequent application of PY-PB and other pyrethroids was demonstrated on many farms in 1973 (Keiding, 1976) and later. However, on farms with aerosol treatments at a week's interval or more low resistance to pyrethroids did not increase generally and the aerosol treatments continued to be effective. However, once a fly population had developed pyrethroid resistance this would in many cases remain high the following year(s) even if the frequency of the aerosol treatments was reduced. If other insecticides were used, pyrethroid resistance might reverse. Based on these results we recommended that the frequency and extent of using pyrethroid aerosols or space sprays should be reduced to decrease the selection pressure by allowing more unexposed flies to reproduce. Moreover, based on the long experience with residual sprays, we persuaded the authorities and companies not to register residual pyrethroid formulations for fly control on farms. The result is that the aerosols are still effective on most Danish farms in spite of the general high potential for development of pyrethroid resistance. The lack of resistance development is illustrated in Table 1 by results of recent surveys of Danish farms.

Table 1.

Cumulative (percent) distribution of resistance ratios (R/S) to bioresmethrin - PB (1:5) in Danish housefly populations monitored in 1973 before and after trials with frequent application of aerosols, and from 1977 to 85 through annual surveys of resistance. Resistance ratios are based on topical application tests.

	Trial		Surveys							
	1973 PRE	1973 POST	1977	1978	1979	1980	1981	1982	1984	1985
No. of farms:	16	15	29	26	33	20	19	19	16	12
R/S at LD_{50}										
< 2.5	19	0	48	69	61	65	63	47	50	58
< 5	100	7	86	92	91	90	95	89	94	100
< 10		47	90	96	97	95	100	95	100	
< 20		87	97	100	100	95		100		
< 40		87	100			100				
< 80		100								
R/S at LD_{95}										
< 2.5	6		21	35	27	40	32	16	25	25
< 5	69		38	77	61	65	63	42	69	75
< 10	100	0	62	85	82	75	75	68	88	75
< 20		13	82	96	91	90	95	89	100	92
< 40		54	93	100	100	95	100	100		100
< 80		87	97			100				
< 160		100	97							
< 320			100							

Combined use of bait and larvicide

The idea is to carry out fly control with two unrelated insecticides and application methods, both used at a moderate rate, in order to reduce selection pressure by either. This principle is practised by many Danish farmers who use methomyl or azamethiphos bait and the insect development inhibitor cyromazine as a larvicide. Trials carried out by DPIL are presently in progress on 9 farms. Three strategies involving azamethiphos bait and cyromazine larvicide are being compared: (1) azamethiphos applied only on strategic places and supplemented with larviciding with cyromazine if necessary, (2) azamethiphos applied abundantly as the only fly control measure, (3) cyromazine larvicide applied to all presumed breeding places. So far housefly larvae on Danish farms have shown complete susceptibility to cyromazine, but the occurrence or potential for resistance to azamethiphos is common. It is hoped that strategies (1) and (3) will reduce the development or persistence of such resistance compared to strategy (2). The results from the first season agree with this expectation (Jespersen et al. 1986).

CONCLUSIONS

Five methods or strategies for fly control used on Danish farms 1953-86 have been discussed: (1) selective residual spraying of 30 or 10% of sprayable surface, (2) use of impregnated ribbons suspended under the ceiling, (3) use of paint-on baits, (4) use of non-residual space spray and (5) combining the use of bait and larvicide. These methods were developed and tried because residual sprays failed due to development of resistance. The aim was to obtain effective and economic fly control with a minimum of resistance problems by reducing the selection pressure through strategic or selective treatments based on studies of housefly biology, behaviour and distribution. This was done either by restricting the insecticide to limited strategic places, as in methods (1), (2) and (3), limiting the frequency of treatments with non-residual treatments (4) or combining two unrelated types of application and insecticide (5). Method (2)-(5) have worked, but selective residual spraying did not reduce development of resistance.

This review summarizes the experience gained from trials and practical control treatments on farms over a long period. In England Denholm et al. (1983, 1985) have analysed the development and reversal of resistance in a fly population on a single pig farm and investigated the importance of gene flow and overwintering to resistance in a group of farms. We consider that our extensive practical experience and the work of Denholm et al. complement each other well and should be included in considerations of resistance management together with the theoretical models of development of resistance. Further analyses of factors influencing resistance development on Danish farms, based on available data on fly control with different methods, on population dynamics and development of resistance and on additional special field experiments, should shed greater light on how best to combat the resistance problem.

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EFFECT OF DIFFERENT INSECTICIDES ON THE SELECTION AND CONTROL OF HIGHLY RESISTANT *MYZUS PERSICAE*

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ABSTRACT

Field cages enclosing rows of potatoes were infested with mixtures of S (susceptible) R_1 (moderately resistant) and R_2 (highly resistant) clones of *Myzus persicae*. Three treatments of pirimicarb (carbamate), demeton-S-methyl (organophosphate) or a deltamethrin/heptenophos mixture (pyrethroid/organophosphate) were applied to plots infested with one of two different starting frequencies of R_2's (0.02 and 0.20). Frequencies of the three variants and the numbers of adults and nymphs present were determined pre-treatment and after each treatment. Resistance was assessed in individual aphids by an immunoassay measuring the activity of E4, the enzyme responsible for resistance. From the 0.02 R_2 starting frequency all three treatments produced R_2 frequencies approaching 1.00 after three sprays. However, the pyrethroid mixture selected more rapidly for highly resistant aphids than the carbamate or organosphosphate. Overall control of aphid numbers is considered in relation to starting frequency and rate of selection for R_2's and implications for field control discussed in the light of R_2 frequencies presently found on field crops.

INTRODUCTION

Insecticide resistance in *Myzus persicae* is conferred by the increased production of the carboxylesterase (E4) which both degrades and sequesters carbamate, organophosphorus (OP) and pyrethroid insecticides (Devonshire & Moores 1982). Biochemical studies have shown that carbamates are hydrolysed less readily than OP's (Devonshire & Moores 1982) and lower resistance to carbamates than to OP's or pyrethroids has also been shown by leaf dip bioassays (Sawicki & Rice 1978). Thus carbamates should select least strongly by preserving a larger proportion of more susceptible aphids in populations under selection. However this prediction has not been tested in the field under the repeated applications advised to prevent the spread of aphid-borne viruses in seed potato crops.

As well as determining the rates at which different classes of insecticide are likely to select for resistance it is important to relate resistance frequencies to the level of control achieved (Denholm *et al.* 1984).

The development of a rapid immunoassay technique capable of determining the resistance levels of large numbers of aphids (Devonshire & Moores 1984, Devonshire *et al.* 1986) has facilitated large scale field trials to establish rates of selection for resistance and the corresponding levels of control achieved by different insecticides.

MATERIALS AND METHODS

Design of field experiment

The three clones used, S (susceptible), R_1 (moderately resistant) and R_2 (highly resistant), originated from field populations in the UK (ffrench-Constant *et al.* in press) and were kept on excised potato leaflets in small plastic boxes (Blackman 1971). In order to provide sufficient numbers for field cage infestation 100 aphids were transferred to three nicotine-fumigated Chinese cabbage plants in population cages. Two different R_2 starting frequencies were established: 0.02 (2 R_2, 49 R_1 and 49 S) and 0.20 (20 R_2, 40 R_1 and 40 S). Populations were left to build up for a fortnight (one week in the glasshouse and another in a sheltered position outside).

Two field cages (metal frame and terylene netting: 3m long, 0.75 m high and 0.5m wide), one for each starting frequency, were erected over two of the rows of potatoes in 12 m x 4.5 m plots (6 rows). Field cages were infested by cutting the leaves from the Chinese cabbage plants in the population cages and placing them on the growing plants.

The twelve plots (planted 24 April 1985), separated by a 7.5 m fallow surround, were laid out in three blocks of four and the treatments, pirimicarb (carbamate), demeton-S-methyl (OP), deltamethrin/heptenophos (pyrethroid/OP mixture) and an unsprayed control, randomised within blocks. The following formulations and rates were used: Aphox (50% pirimicarb dispersible granules; ICI) sprayed at 140 g ai/ha, Metasystox 55 (58% demeton-S-methyl emulsifiable concentrate; Bayer) sprayed at 244 g ai/ha and Decisquick (2.5% deltamethrin and 40% heptenophos emulsifiable concentrate; Hoechst) sprayed at the equivalent of 7.5 and 120 g ai/ha respectively. Each treatment was sprayed three times at 14 day intervals (25 June, 8 and 23 July).

Sampling and resistance monitoring

Cages were sampled pre-treatment, and one and eight days after each spray (the relationship between sample number and times of spraying is shown in Fig. 1).

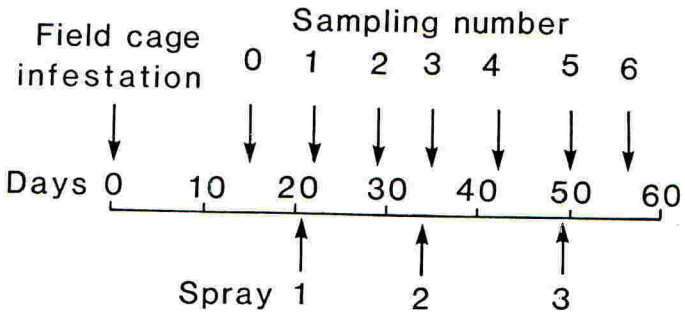

Fig. 1. The relationship between times of field cage infestation, spraying and sampling number.

Thirty leaves were removed, one from the top, middle and bottom of each of the ten plants in a cage, individually enclosed in polythene bags and taken back to the laboratory. The total numbers of adults and nymphs per leaf were estimated to the nearest five. Eighty-four adult apterae removed at random for resistance screening were placed in 50 μl of PBS/Tween in the wells of a 96-well immunological plate. Four aphids from each of the three standard laboratory clones were placed in the remaining 12 wells and the plates immediately frozen at -20°C. The frequencies of the resistance variants were determined by immunoassay (Devonshire *et al.* 1986); all plates were analysed within a week of collection, having established that storage did not influence results.

All data were logarithmically transformed and separate analyses of variance performed on control and treatment data for both resistance frequencies and aphid numbers; the full results of these analyses are presented elsewhere (ffrench-Constant *et al.*, in press). In this paper S and R_1 aphid frequencies and numbers have been pooled to simplify presentation and the mean number of aphids sampled from each plant of a treatment was calculated by summing the three leaf positions for individual plants and averaging over the 30 plants from the three blocks.

RESULTS

When populations were sampled from field cages before any treatment (i.e. sample number 0), R_2 aphids had increased from their initial starting frequencies of 0.02 and 0.20 to 0.15 and 0.31, respectively. Proportions of R_2 aphids continued to increase initially in control plots, but then reverted to levels approximating the initial starting frequencies (Fig. 2a,b).

Following treatment, the proportions of R_2's increased rapidly from the 0.02 starting frequency and approached 1.00 after three sprays for all chemicals. This increase was most rapid with the deltamethrin/heptenophos mixture (Fig. 2a).

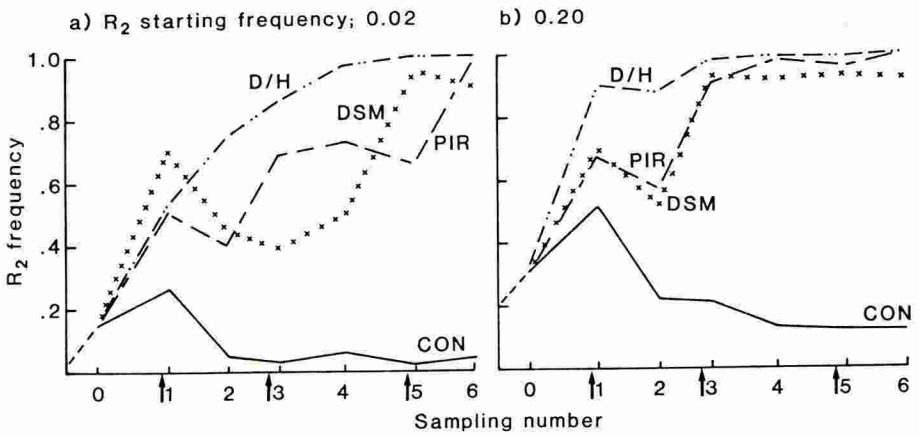

Fig. 2. Change in R_2 frequency over time in plots untreated (CON), or treated with pirimicarb (PIR), demeton-S-methyl (DSM) or deltamethrin/ heptenophos (D/H).

a) 0.02
CON
PIR
DSM
D/H
1000
100
10
0
Log (n+1)
S+R1
R2
0 1 2 3 4 5 6
b) 0.20
Sampling number

Fig. 3. (Opposite) Mean total numbers of aphids sampled/plant (— — —) in cages untreated (CON) or treated with pirimicarb (PIR), demeton-S-methyl (DSM) or deltamethrin/heptenophos (D/H) with 0.02 and 0.20 R_2 starting frequencies. Stippled areas denote estimated numbers of R_2's.

Increasing the starting frequency to 0.20 increased the rate at which R_2 frequencies approached 1.00 (sample 3) and decreased the persistence of S and R_1 variants observed with pirimicarb and demeton-S-methyl (Fig. 2b).

Mean numbers of aphids counted/plant and estimated numbers of R_2's under the different treatments for the two R_2 starting frequencies are shown in Fig. 3a and b (numbers of R_2's were calculated as the product of total numbers and R_2 frequency). Total numbers in the control showed a rapid increase followed by a decline, which was less pronounced in the 0.20 R_2 starting frequency cages.

All treatments reduced peak numbers of aphids at the 0.02 R_2 starting frequency. However final levels of control achieved differed in relation to the rates at which R_2's were selected by the different chemicals. Thus, progressively more aphids were found in the pirimicarb, demeton-S-methyl and deltamethrin/heptenophos cages, the increase being inversely related to the rates of selection for R_2's observed. Increasing the R_2 starting frequency to 0.20 reduced the level of control achieved by the chemicals and decreased the differences between treatments, reflecting more rapid and uniform rate of selection for R_2's.

Separate analyses of numbers of adults and nymphs showed that the higher numbers of aphids in the deltamethrin/heptenophos plots were largely due to the proportionately larger numbers of nymphs present as compared to the other chemicals, e.g. pirimicarb (Fig. 4).

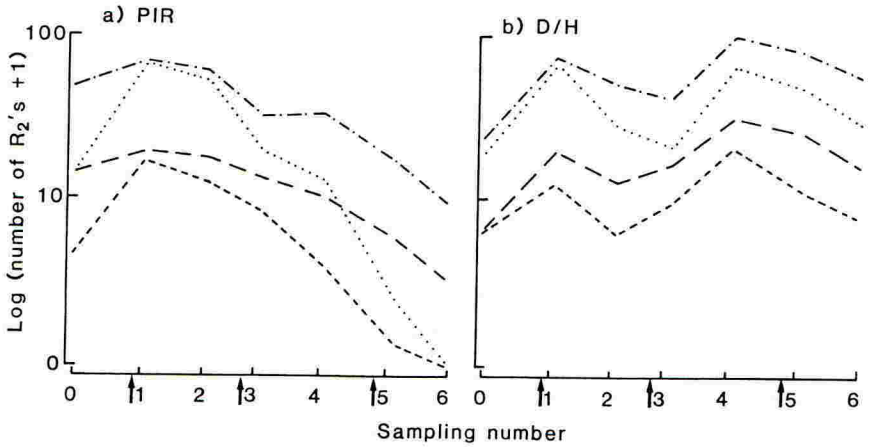

Fig. 4. Estimated mean numbers of R_2 aphids/plant over time in the pirimicarb (PIR) and deltamethrin/heptenophos (D/H) treatments from the 0.02(·····, nymphs; - - - -, adults) and 0.20 (-·-·-, nymphs; — —, adults) R_2 starting frequencies.

DISCUSSION

Rates of selection achieved by the different classes of insecticide broadly confirm predictions from biochemical studies and leaf dip bioassays. Thus where only one application is anticipated pirimicarb should be used in preference to the other insecticides because of its slower selection for highly resistant aphids and better control achieved. However, rapid increases of R_2's on repeated application of all the compounds highlights the need for new classes of insecticide and/or control methods for *M. persicae* as the build up of this variant in an area will seriously threaten effective chemical control.

The proportionately higher numbers of R_2 nymphs in the deltamethrin/heptenophos plots suggest that this insecticide stimulated the production of nymphs. This phenomenon has been observed in laboratory experiments with aphids confined on deltamethrin treated glass surfaces (A.D. Rice unpublished data). In the field, Foster (1986) observed a nine-fold increase in total aphid numbers following four sprays of deltamethrin/heptenophos in unconfined field trials. The effects of rapid R_2 selection and stimulated nymph production by pyrethroid/OP mixtures will be studied further in the light of their recent introduction for virus control in potatoes and sugar beet.

Although the presence of resistance in aphid populations may not enhance their ability to spread non-persistent viruses, such as potato virus Y, which are transmitted by the probing of many species landing temporarily on the crop (Harrington *et al.* 1986), R_1 aphids have undoubtedly contributed to the increased spread of potato leaf roll virus (a persistent virus, requiring long periods of feeding for transmission) on treated plots (Foster *et al.* 1981).

Under open field conditions rates of selection may be slowed, compared with isolated field cages, by the immigration of unselected aphids following spraying; this may account for the similarity in resistance frequencies sometimes observed in sprayed and unsprayed areas (Furk 1986). However, this dilution cannot be relied upon to delay its build up, as R_2 frequencies up to 0.20 and the virtual absence of S aphids have been found on unsprayed winter rape from a range of sites around southern and eastern England in October 1985 (ffrench-Constant & Devonshire 1986). Previously, high frequencies of R_2's have only been recorded from localised areas of northern England (Furk 1986) and Scotland (Sawicki *et al.* 1978). Control failure associated with frequencies of highly resistant aphids of 1.00 has also been observed in 1986 after repeated use of pyrethroids on uncaged potatoes in field trials (R.H. ffrench-Constant unpublished data). Thus studies such as this are necessary both to monitor the development of resistance in the field and to formulate strategies to prevent control failure and prolong the life of existing insecticides.

ACKNOWLEDGEMENTS

We thank S. Clark and J. Perry for help with analysis of the results, M. Stribley for culturing the aphids, and I. Denholm for advice on the manuscript. R.H. ffrench-Constant was supported by an AFRC studentship.

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EFFECTIVENESS OF INSECTICIDE-ADJUVANT MIXTURES AGAINST SUSCEPTIBLE AND MONOCROTOPHOS-RESISTANT STRAINS OF *SPODOPTERA LITTORALIS* IN THE LABORATORY

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ABSTRACT

The potential use of four oil/surfactant mixtures ('Atplus' 411F, 412, 415 and 417, Imperial Chemical Industries PLC) as adjuvants to increase the efficacy of insecticides against resistant insects was assessed from laboratory bioassays on third instar larvae of susceptible and monocrotophos-resistant strains of *Spodoptera littoralis* Boisd..In most cases the addition of these mixtures (3000 ppm) to solutions of methomyl, diflubenzuron, monocrotophos and profenophos led to an increase in mortality over that obtained with the insecticide alone. This increase was greatest with methomyl and diflubenzuron and generally more pronounced in the resistant strain. The findings indicate a possible role for adjuvants in controlling resistant individuals, but much more extensive testing and field trials are required to assess their value for combatting resistance under field conditions.

INTRODUCTION

The use of vegetable or mineral oils, together with surfactants to enhance the effects of herbicides, is well known (Gillard 1985), but no references to such effects with insecticides could be found. Mixtures of insecticides and oils increased efficacy of the insecticides (Johnson *et al.* 1978, House *et al.* 1980, Singh *et al.* 1981, Castellani *et al.* 1984).

The aim of this study was to assess the ability of four potential adjuvants to increase the efficacy of four insecticides against third instar larvae of a susceptible and a monocrotophos-resistant strain of *Spodoptera littoralis* Boisd.

MATERIALS AND METHODS

Insecticides

Four insecticides - monocrotophos and profenofos (organophosphorous insecticides, methomyl (carbamate) and diflubenzuron (chitin synthesis inhibitor) - were applied alone or with adjuvants as technical grade insecticides dissolved in water.

Adjuvants

Four potential adjuvants ('Atplus'-type, Imperial Chemical Industries PLC) are designated by code numbers thus: 411F and 415-mixtures of nonphytotoxic paraffinic mineral oil with 17% and 5% respectively of surfactant 300F; 412 and 417-mixtures of rape-seed oil with 17% and 5% respectively of the surfac-

tant blend 298/299.

Insects

S. littoralis consisted of a susceptible (yellow-eyed) and a resistant (brown-eyed) strain to monocrotophos (Dittrich & Luetkemeier 1980). The insects were reared on castor bean leaves as described by El-Defrawi *et al.* 1964. All stages were kept at 23°C, 55% R.H., and a 16 h photoperiod. Each generation of the resistant strain was exposed during the 3rd larval instar to monocrotophos-dipped leaves at 0.4 g a.i./l.

Insecticide testing

Initial bioassays were performed to establish LC_{25} and LC_{50} for each insecticide alone on 3rd instar larvae of the S- and R strain using the dipping method (Auda & Degheele 1985). Each of the four adjuvants was added (3000 ppm) to solutions of each insecticide at concentrations corresponding to calculated LC_{25s} and LC_{50s} for the S- and R strains.

Mortality due to these combinations was established after 24 h for monocrotophos, profenofos and methomyl and after ecdysis for diflubenzuron (larvae being fed every day on treated leaves). Four replicates each of ten larvae were used, mortality being corrected according to *Abbott*'s formula (Abbott 1925).

RESULTS

Insecticides alone

LC_{25} and LC_{50} values for the four insecticides alone against both *S. littoralis* strains are listed in Table 1.

TABLE 1.

LC_{25} and LC_{50} values (ppm) of the insecticides to 3rd instar larvae of S- and R strains of *S. littoralis* (Auda & Degheele 1985)

Insecticide	S strain		R strain		Resistance Ratio*
	LC_{25}	LC_{50}	LC_{25}	LC_{50}	
profenofos	59	79	400	510	6.5
monocrotophos	150	280	16,000	40,000	150
methomyl	29	49	150	210	4.3
diflubenzuron	8.1	12	19	35	2.8

* $RR = \frac{LC_{50} \text{ of resistant strain}}{LC_{50} \text{ of susceptible strain}}$

The R strain showed 150-fold resistance to monocrotophos. This strain was also slightly more tolerant to the other insecticides in comparison with the S strain.

Insecticides + adjuvants

No toxicity was observed when leaves were treated with adjuvants alone either at low (50 ppm) or at high (10,000 ppm) concentrations.

Addition of the four adjuvants to methomyl resulted in increased mortality at both levels of toxicity (LC_{25} and LC_{50}) for the insecticide alone (Fig. 1.). Similar increases were obtained with diflubenzuron (Fig. 2.).

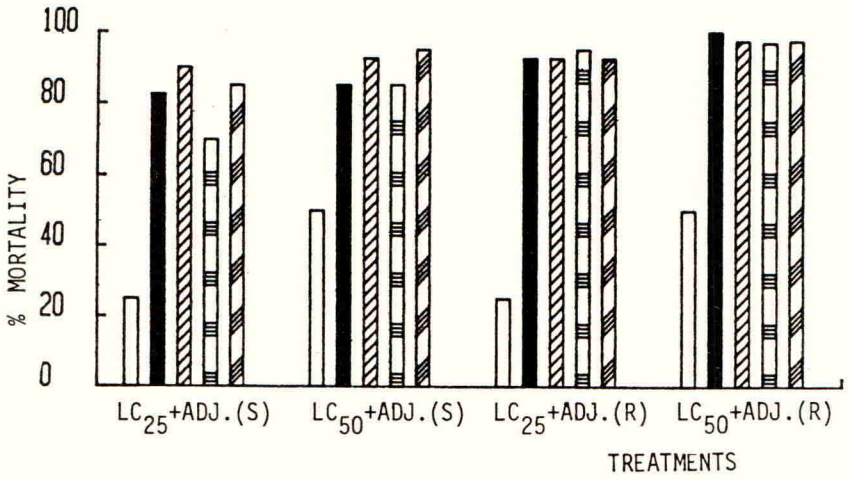

Fig. 1. Mortality with methomyl of the S- and R strains resulting from the addition of adjuvants to concentrations corresponding to the LC_{25} and LC_{50} values for the insecticide alone. Key to symbols: □ insecticide alone, ■ insecticide + 411F, ▨ insecticide + 412, ▬ insecticide + 415, ▨ insecticide + 417.

Results obtained with the organophorous insecticides were more variable (Fig. 3 and 4). 412, 415 and 417 increased mortality with monocrotophos very substantially in both strains. 411F was effective against the R strain, but had little effect against the S strain. 415 and 417 were most effective in combination with profenofos in both strains.

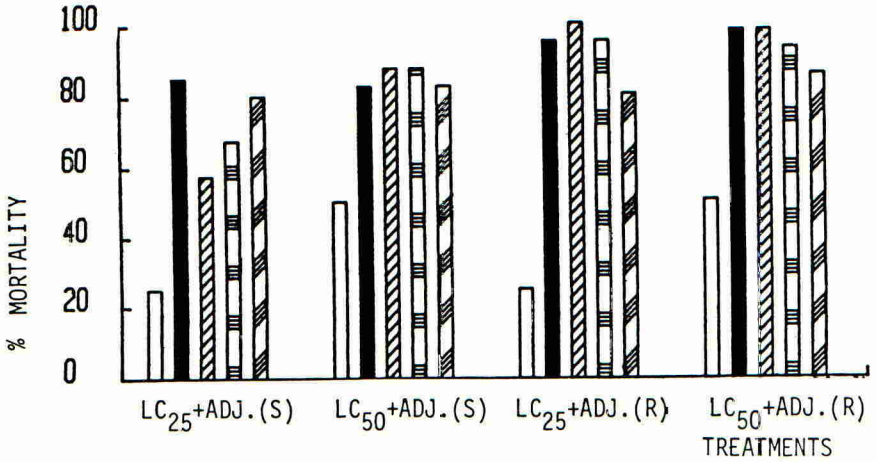

Fig. 2. Mortality with diflubenzuron of the S- and R strains resulting from the addition of adjuvants to concentrations corresponding to the LC_{25} and LC_{50} values for the insecticide alone. Key to symbols: ☐ insecticide alone, ■ insecticide + 411F, ▨ insecticide + 412, ▬ insecticide + 415, ▨ insecticide + 417.

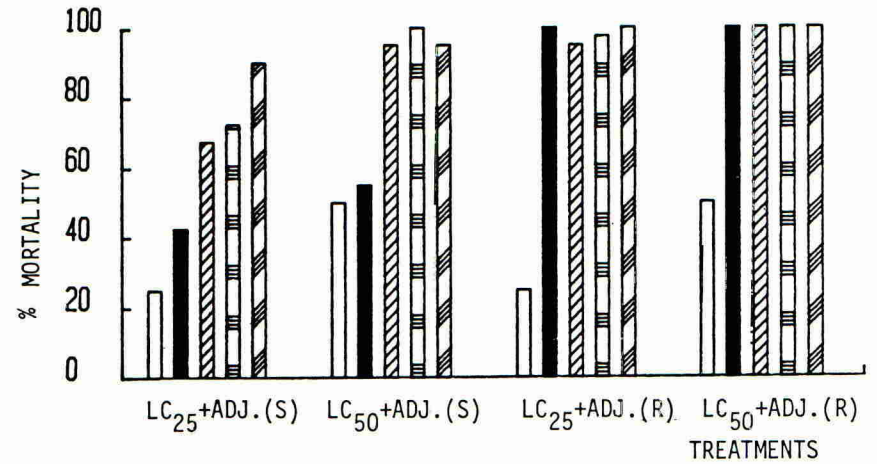

Fig. 3. Mortality with monocrotophos of the S- and R strains resulting from the addition of adjuvants to concentrations corresponding to the LC_{25} and LC_{50} values for the insecticde alone. Key to symbols: ☐ insecticide alone, insecticide + 411F, ▨ insecticide + 412, ▬ insecticide + 415, ▨ insecticide + 417.

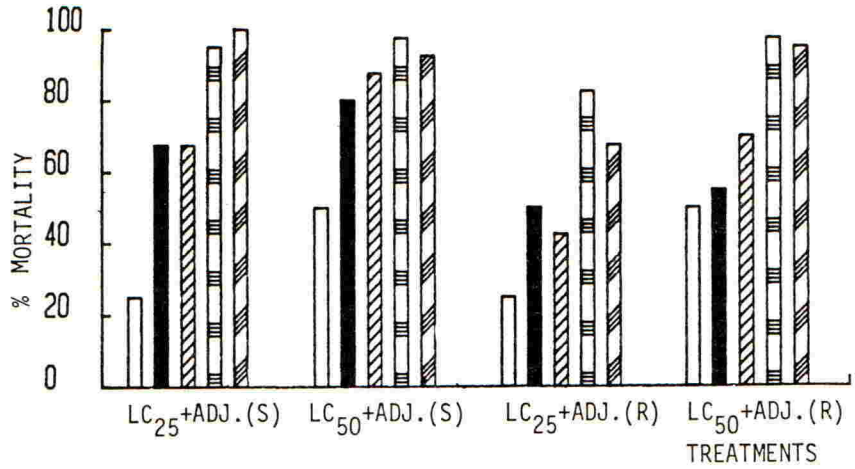

Fig. 4. Mortality with profenofos of the S- and R strains resulting from the addition of adjuvants to concentrations corresponding to the LC_{25} and LC_{50} values for the insecticide alone. Key to symbols: ☐ insecticide alone, ■ insecticide + 411F, ▨ insecticide 412, ▤ insecticide + 415, ▨ insecticide + 417.

DISCUSSION

Whatever the underlying causes, the results reported indicate that insecticide and adjuvant combinations offer some potential for regaining control of R insects poorly controlled by the insecticide alone. However these findings must be interpreted with caution because the superiority of adjuvants in this respect can only realistically be assessed in comparison with formulations currently in use for pest control in the field. Extensive field testing will be required to evaluate the addition of adjuvants as a viable resistance-countering tactic.

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THE RESPONSE OF RESISTANT HOUSEFLIES TO AN UNSYNERGISED AND SYNERGISED N-ALKYLAMIDE

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ABSTRACT

An insecticidally active N-alkylamide (5-bromo-2(6-(1,2 dimethyl-propylamino)-6-oxo-hexa-2(E),4(E)-dienyl)naphthalene, BTG 502) was bioassayed without and with piperonyl butoxide against adults of twenty insecticide resistant populations of field-collected houseflies (*Musca domestica* L.). Piperonyl butoxide strongly synergised BTG 502 even against the susceptible strains and eliminated the BTG 502 resistance shown by most of the populations tested except in one strain which very strongly resisted (> 500 fold) the combination of BTG 502 plus piperonyl butoxide.

INTRODUCTION

Recent interest in N-alkylamides has led to the discovery of insecticidally active compounds (Elliott *et al.*, in press). Some of these compounds have shown remarkable negatively correlated cross-resistance in pyrethroid-resistant adult housefly strains in which the only resistance factor is super-kdr-3D (Elliott *et al.* 1986).

The present study examines the effects of an insecticidally active N-alkylamide (BTG 502) to which there is negatively correlated cross-resistance in flies with super-kdr-3D, in populations of housefly which have been collected in the field in several countries and which have various factors of resistance.

MATERIALS

Housefly strains

The twenty strains of housefly which had been collected originally from the field are listed in Table 1 along with their sources. The standard susceptible Cooper strain has been reared in this laboratory for more than thirty years without contact with insecticides.

Chemicals

The two compounds used in bioassays were 5-bromo-2(6-(1,2-dimethyl-propylamino)-6-oxo-hexa-2(E),4(E)-dienyl)napthalene (BTG 502) which was synthesised in this Department, and piperonyl butoxide. Both compounds were >95% pure.

METHODS

Female houseflies, three to five days old were each treated with a solution of the insecticide in acetone (0.5 µl/fly) with two replicates of 15 flies per dose. Treatment and post-treatment were at 20°C, and kill was assessed 48 h after application. Piperonyl butoxide (1.0 µg/fly) was applied with BTG 502. For most strains, bioassays were repeated several times and weighted mean LD_{50} values are presented in µg BTG 502/fly.

RESULTS

Response of different housefly strains to BTG 502

BTG 502

Strain 17bb, known to have only kdr was, as expected, (Elliott *et al.* 1986) about 2-3 times more susceptible than the standard Cooper strain as was strain 228e2b (Fig. 1). The LD_{50}s of eleven of the twenty strains tested ranged between 0.15 μg/fly (the LD_{50} for the Cooper strain) and 1.3 μg/fly. Of the remaining seven with LD_{50}s greater than 1.9 μg/fly (RF >17), two (strains 39m2b and AIN) were virtually immune even to doses as strong as 10 μg/fly.

BTG 502 + piperonyl butoxide

Pb synergised BTG 502 10-27 fold against those strains whose LD_{50} for the compound alone did not exceed 1.3 μg/fly (the only exception being Canada, LD_{50} 1.0 μg/fly, SF = 50) (Fig. 2). For the other strains SFs increased with increasing LD_{50} and exceeded 600 for strain 39m2b (Fig. 3). The one strain that did not conform was AIN against which synergised BTG 502 was almost ineffective.

DISCUSSION

Cross-resistance characteristics of the strains bioassayed with BTG 502

The twenty strains used here had been tested systematically for resistance to pyrethroids, and for the presence of kdr or super-kdr and pen, the resistance mechanism which delays cuticular penetration. We tested a few strains for resistance to azamethiphos; some of the other strains were tested for resistance to several organophosphorus insecticides by J. Keiding (personal communication) and P.R. Carle (personal communication).

There was no correlation between multifactorial resistance to pyrethroids and BTG 502. The tolerance to BTG 502 of the pyrethroid resistant strains, many heterozygous for kdr or super-kdr, ranged from almost complete susceptibility to virtual immunity. Of the two strains most resistant to BTG 502, AIN very strongly resisted pyrethroids while 39m2b was almost fully susceptible to these insecticides. Only strain 17bb which has no known resistance mechanisms other than kdr, showed negative cross-resistance to BTG 502.

Although there was little apparent correlation between the resistance to BTG 502 and the penetration delaying factor pen in strains heterozygous for this factor, the two strains most resistant to BTG 502 (AIN and 39m2b) were also exceptionally resistant to tributyltin acetate, the diagnostic indicator for pen.

There was no apparent cross-resistance between BTG 502 and azamethiphos or trichlorphon, but the two strains most resistant to BTG 502 also resist dimethoate.

CONCLUSIONS

From the available data it can be assumed that LD_{50} values between 0.15 and 1.0 μg/fly BTG 502 most probably represent the normal range of responses to this compound in field populations of houseflies, while those in excess of 2 μg/fly denote resistance which is readily suppressed by piperonyl butoxide. Indeed, piperonyl butoxide is an extremely effective

synergist for BTG 502 even in susceptible strains, a possible sign that the insecticide can be readily detoxified presumably by pb-suppressible microsomal metabolism.

There is however additional resistance to BTG 502, so far only observed in the AIN strain, that is not suppressible by pb, and whose nature is unknown. Moreover the very strong resistance through pen to tributyltin acetate in strains AIN and 39m2b suggests that delayed penetration may interact with the two BTG 502-resistance mechanisms.

The negative correlation between pyrethroid resistance and BTG 502 only works when kdr or super-kdr are the sole pyrethroid resistance mechanism present (e.g. in strain 17bb) since in all other pyrethroid resistant strains tested in which resistance is almost certainly multifactorial there was no detectable negative cross-resistance.

As yet, it has not been possible to identify the cross-resistance characteristics which select for resistance to BTG 502. It is interesting to note however that the two most resistant strains were both multi-resistant, including strong resistance to dimethoate, but only 39m2b had a DDT-resistance that is suppressible by piperonyl butoxide on chromosome V (Sawicki & Farnham, 1969).

Our results demonstrate that although a number of housefly strains from several countries resist BTG 502, in almost all cases, the addition of piperonyl butoxide eliminates this resistance completely.

ACKNOWLEDGEMENTS

We gratefully acknowledge the gift of various strains of houseflies used in this study from J. Keiding, P.R. Carle, C.R. Harris and R. de Deken.

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TABLE 1

List of housefly strains tested for present study.

Strain	Origin	Comment
17bb	Denmark	Occasional lab-selection with DDT since 1950.
$228e_2b$	"	Occasional lab-selection with pyrethrins/pb.
$252e_3$	"	Pyrethroid resistant.
357i	"	Dimethoate resistant. DDT resistance pb suppressible.
$39m_2b$	"	Tetrachlorvinphos resistant.
$49r_2b$	"	Dimethoate resistant.
AIN	France	Multi-resistant to ops, ocs and pyrethroids.
Bugper	Belgium	Permethrin resistant.
Guelph	Canada	" "
Zurich b	Switzerland	" "
NPR	Sweden	Lab-selection with natural pyrethrins.
Fm 3	England	Strongly lab-selected with permethrin.
Fm 22	"	" " " "
Fm 44	"	)
Fm 55	"	)
Fm 59	"	) These strains were collected from
Fm 60	"	) farms in the UK during a recent
Fm 61	"	) survey to estimate the distribution
Fm 62	"	) and levels of insecticide resistance.
Fm 63	"	)

Information about housefly strains from outside UK kindly provided by J. Keiding, P. Carle, C. Harris and R. de Deken.

Figure 1. Distribution of LD50s of 18 field strains for BTG 502

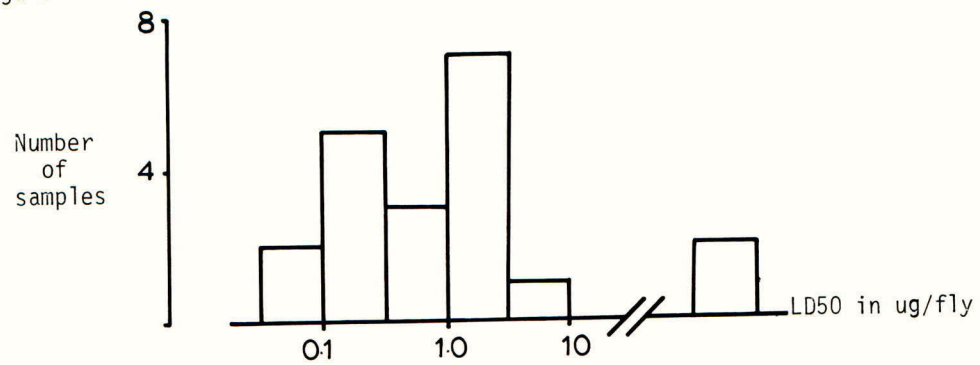


Figure 2. Distribution of LD50s of 18 field strains for BTG 502 + piperonyl butoxide

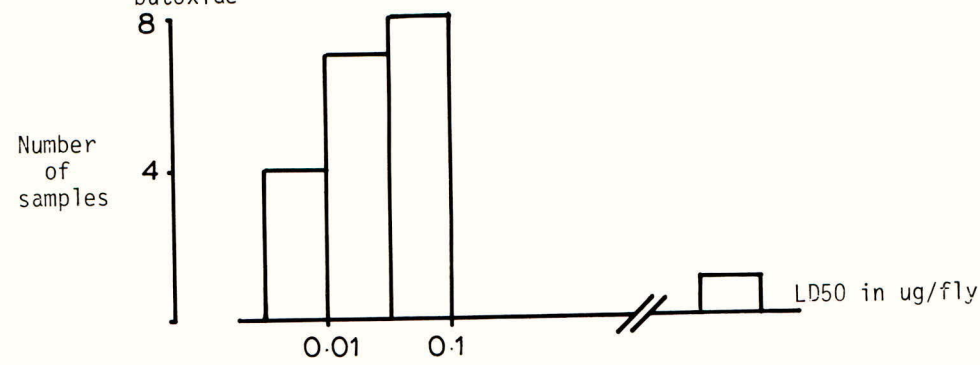
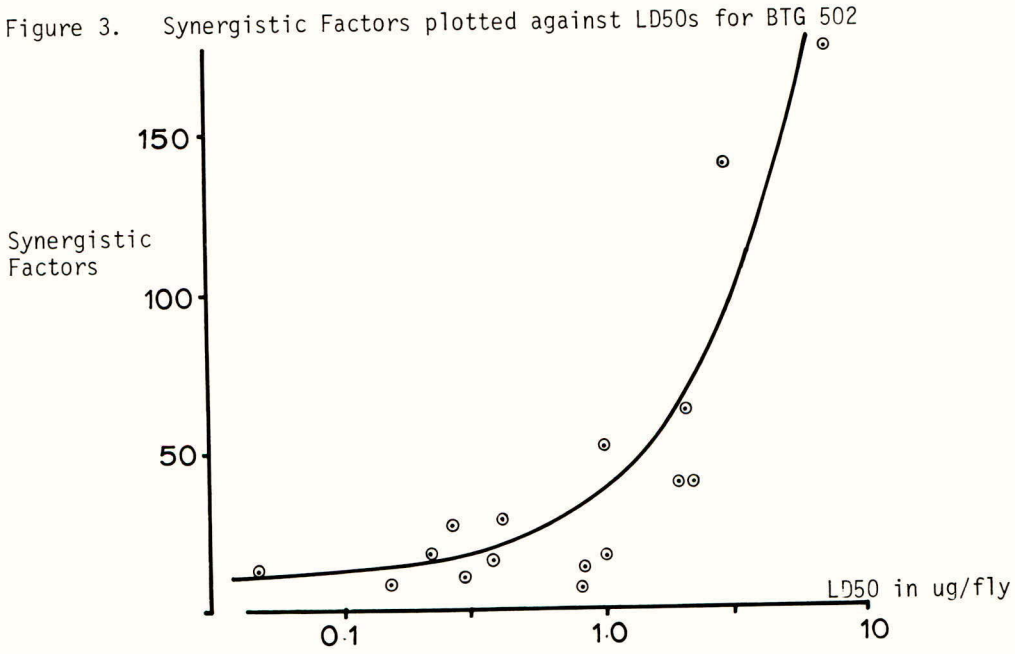


Figure 3. Synergistic Factors plotted against LD50s for BTG 502



RESISTANCE AND THE COLORADO POTATO BEETLE IN NEW JERSEY: PLANT-PESTICIDE INTERACTIONS

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ABSTRACT

In laboratory studies, Colorado potato beetle (CPB) (Leptinotarsa decemlineata Say) larvae reared on eggplant were significantly more susceptible to a pyrethroid (permethrin) insecticide and to permethrin plus piperonyl butoxide as compared to larvae reared on tomato. Field studies conducted in 1984 and 1985 showed that percent reduction of CPB larvae reared on eggplant due to foliar applications of a pyrethroid insecticide (fenvalerate) was significantly greater than percent reduction of CPB larvae reared on either potato or tomato. Effect and biological activity of individual glycoalkaloids in various Solanum species need to be examined in detail before the full significance of the role of glycoalkaloids in host plant resistance and CPB insecticide resistance is understood.

INTRODUCTION

The Colorado potato beetle (CPB), Leptinotarsa decemlineata (Say), is a severe pest of eggplant (Solanum melongena L.), white potato (S. tuberosum L.) and tomato (Lycopersicon esculentum Mill.) production in New Jersey. The CPB has the potential to completely defoliate these crops as well as directly damage the fruit.

The CPB was removed from pest status with the introduction of DDT in the 1940's, but quickly developed DDT resistance and became a limiting factor in the production of these crops (Hofmaster 1956). Over the years, the CPB has also developed resistance to virtually all classes of insecticides used against it as a succession of organochlorine, cyclodiene, organophosphate and carbamate insecticide resistance occurred in the northeastern U.S. (Gauthier et al. 1981). The problem of CPB management is currently compounded by pyrethroid resistance (Forgash 1981) because growers now face highly resistant CPB populations with marginally effective insecticides and few prospects for new, more effective compounds.

Insecticide synergists are potentially important pest management components as they may increase the insecticidal activity against resistant arthropods. Laboratory and field studies have shown that piperonyl butoxide synergizes both permethrin and fenvalerate (Ghidiu and Silcox 1984, Silcox

et al. 1985), and that synergized pyrethroids provided significantly greater control of the CPB than did comparable dosages of the pyrethroids alone.

From resistance studies with insect species other than CPB, known resistance mechanisms are enhancement of biochemical and physiological barriers to intoxication, decrease in sensitivity of the target site, and modification of behavior leading to reduced exposure to the toxicant (Georghiou 1972, Forgash 1981). Of these, metabolic detoxication is the preeminent means whereby insects resist insecticides. Forgash (1981) further reports that resistance to organochloride, organophosphorus and carbamate insecticides appears to be based on the same, or similar, backgrounds of ancillary genes. Thus substitution of one chemical for another within these groups tends to preserve or even enhance the existing resistance.

It is strongly suspected that some of the enzymes the CPB utilizes to detoxify insecticides are also utilized to break down potentially toxic alkaloids in the foliage of Solanum species (Grafius 1985). Steroidal glycoalkaloids produced by many Solanum species are toxins or potent feeding deterrents (Buhr et al. 1958), and an important function of the glycoalkaloids is considered to be plant protection. To survive on these plants, then, the CPB must be able to detoxify these glycoalkaloids through the action of enzymes it produces. Certain wild species of potatoes are less susceptible to CPB damage than cultivated potatoes (Hsiao 1974, Sinden et al. 1980), and the observed resistance has been attributed in part to the presence of steroidal glycoalkaloids in the plant tissue. Similarly, some accessions of tomato plants are highly resistant to CPB feeding whereas the cultivated tomato is suspectible. Sinden et al. (1978) reported that tomatine content in tomato plants and CPB feeding rate were negatively correlated.

It is tempting to speculate that variations of glycoalkaloids in solanaceous plants are not only responsible for host plant resistance, but may also be responsible for predisposing the CPB to a higher level of insecticide resistance. Sturckow and Low (1961) concluded that beetles resistant to DDT are significantly less sensitive to glycoalkaloids than are normal susceptible beetles.

The CPB, then, would obtain a natural enhancement of its ability to detoxify insecticides through the action of enzymes it has at its disposal as a mechanism to detoxify glycoalkaloids.

The objectives of our investigation were to (a) determine in the laboratory if CPB reared on tomato and eggplant were equally susceptible to pyrethroids, and (b) to conduct field tests with CPB on tomato, potato and eggplant to determine if host plant influences beetle control.

MATERIAL AND METHODS

Laboratory study

During August of 1983, 4th instar CPB larvae were collected from both untreated eggplant (S. melongena L.) and tomato (L. essulentum Mill.) at

Rutgers Research and Development Center in Bridgeton, NJ. Beetles were collected 24 h before each test and were maintained on either eggplant or tomato foliage at 18°C.

Test concentrations were obtained by diluting technical fenvalerate (93.6%), permethrin (93%) and PB (90%) with re-agent grade acetone. The dilutions were prepared in geometric progression 36 h before each test and were stored at -18°C until being brought to room temperature for application. Fenvalerate and permethrin were applied individually to determine dosage-mortality lines for nonsynergized treatments. Synergism was determined by applying fenvalerate or permethrin at a 1:4 ratio (insecticide: PB) with PB being applied as a 2 h pretreatment (Forgash 1981). Treatments were applied to 3 replicates of 10 beetles (30 beetles/concentration). Carbon-monoxide anesthetized beetles received treatment in 2-µl droplets from an ISO microapplicator to the 3rd abdominal segment of larvae.

Larvae were maintained in 9 cm rearing dishes at 22°C, and mortality was recorded at 72 h. Log dosage-probit lines were determined, after correcting for control mortality, by the SAS computer program. Between crop comparisons were made to determine host plant influence on larval toxicity.

Field studies

Potato, tomato and eggplant were planted to a sassafras sandy loam field. In 1984, 'Katahdin' potato were planted on April 12 and 'Rutgers 39VF' tomato and 'Harris Special Hibush' eggplant were transplanted to the field on May 22. In 1985, the same varieties were used for the test, and potato were seeded on April 19 and tomato and eggplant were transplanted on May 16.

All rows were 7.62 m long and 1.53 m wide. Treatments were replicated 4 times in a split-plot experimental design: main plots were rate of pyrethroid (0.0, 0.056, 0.112 and 0.224 kg/ha of fenvalerate) and subplots were crops (potato, tomato, eggplant). Fenvalerate (Pydrin 2.4EC) was selected for the field trial treatments since CPB in New Jersey are significantly more resistant to permethrin than to fenvalerate (Forgash 1981, Silcox et al. 1985). Each subplot consisted of 3 rows, and subplots were treated with a tractor-mounted 3-row boom sprayer with one nozzle over the center of each row and a nozzle on either side of the row calibrated to deliver 590 l/ha at 275.8 kPa operated at 3.2 km/hr; all 3 rows in each subplot were treated. Five plants in the middle of the center row of each subplot were marked off with 30.5 cm wooden stakes, and CPB larvae were counted immediately before treatments were applied. Larval counts were again made on the same 5 plants 48 h posttreatment to determine percent larval reduction. In 1984, treatments were applied on June 15, 20, 28, July 9, 21 and August 3; in 1985, treatments were applied on June 20, 25, 27, July 8 and 17.

RESULTS AND DISCUSSION

Laboratory studies

Host plant toxicity data for 4th instar CPB larvae reared on tomato or eggplant are presented in Table 1. Three of the 4 treatments had LD50 values that were nearly identical for larvae from eggplant and tomato

(fenvalerate, fenvalerate + PB, permethrin + PB). Larvae treated with permethrin alone showed significant host plant-dependent LD50 differences. Larvae reared on tomato showed a significantly greater LD50 than those reared on eggplant and this also resulted in a substantially greater SR value for larvae reared on tomato and treated with permethrin plus PB. The LD50 for permethrin was significantly higher on both crops than the LD50 for fenvalerate. These results show that CPB larvae feeding on tomato foliage may be less susceptible to permethrin than those feeding on eggplant, which suggests that tomato phytochemicals may induce enzymes in the CPB to detoxify permethrin.

TABLE 1.

Host plant toxicity data for PB-synergized and nonsynergized fenvalerate and permethrin applied to 2nd generation 4th instar Colorado potato beetle larvae reared on eggplant or tomato at Bridgeton, N.J. in 1983.

Insecticides	Ratio	Host	Slope	LD50[a]	95% Fiducial Limits
Fenvalerate	-	Eggplant	2.81	1.29	1.07-1.60
Fenvalerate	-	Tomato	1.99	1.29	.99-1.78
Fenvalerate + PB[b]	1:4	Eggplant	3.42	.43	.36- .51
Fenvalerate + PB	1:4	Tomato	3.16	.48	.40- .57
Permethrin	-	Eggplant	3.06	3.33	2.66-3.89
Permethrin	-	Tomato	2.81	5.03	4.18-6.71
Permethrin + PB	1:4	Eggplant	3.11	1.26	1.01-1.56
Permethrin + PB	1:4	Tomato	3.43	1.28	1.09-1.52

[a] µg/beetle (insecticide)
[b] PB = piperonyl butoxide (2-h pretreatment)

Field studies

Across all main plots only the high rate of fenvalerate significantly reduced the number of CPB larvae in 1984 and 1985. After correcting for control reduction, the greatest reduction of CPB larvae occurred on eggplant each year (Figure 1 and 2). Reduction of larvae on potato and tomato due to fenvalerate applications was not significantly different among rates between these two crops. Differences among crops in the response of CPB to increasing rates of fenvalerate were also apparent, both in 1984 and 1985, in that the slope of the regression line was steeper for eggplant than for potato or tomato; i.e., the effect of increasing fenvalerate concentration was greater on eggplant. In 1984, when fenvalerate treatments were repeated 3 times over the course of the season, efficacy (averaged over all rates) declined over time for each crop, but the reduction of efficacy was greater for CPB on tomato (from 50% to 8% to 2%) than on potato or eggplant (from 47% to 22% to 17%) (Figure 3). Thus, the lower fenvalerate efficacy on tomato appears to be coupled with greater pressure on the CPB population for fenvalerate detoxication capability, possibly in terms of maximum detoxication efficiency or inducibility. The significant reduction of CPB larvae on eggplant, as compared

to tomato and potato, supports the possibility that plant glycoalkaloids may be responsible for enhancing the CPB's resistance to insecticides. Forgash (1981) demonstrated that a thorough and efficient application is important, where the poorer the foliar coverage, the greater the beetle recovery and survival. However, since both the laboratory tests and field studies resulted in similar differential control of CPB larvae among host plants, it is believed that plant canopy or density of foliage did not have a significant role in these results.

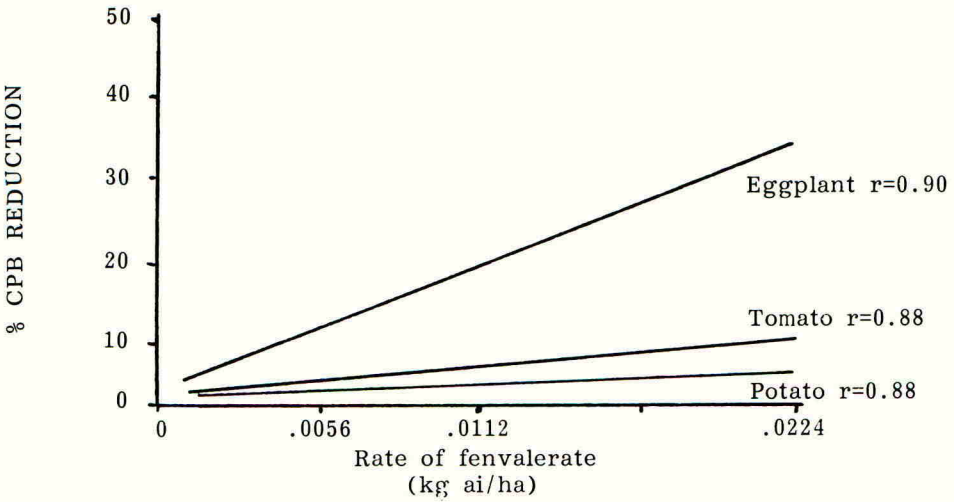

Fig. 1. Percent reduction of Colorado potato beetle larvae on solanaceous crops due to foliar applications of fenvalerate, Bridgeton, N.J., 1984.

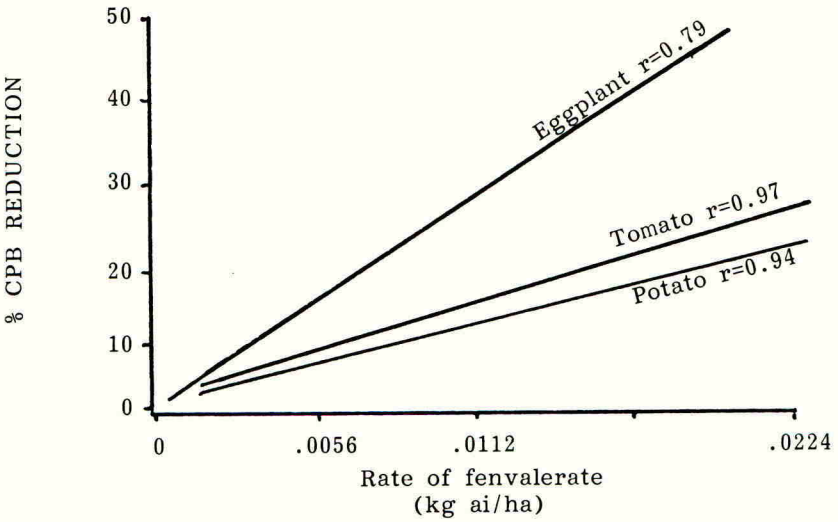

Fig. 2. Percent reduction of Colorado potato beetle larvae on solanaceous crops due to foliar applications of fenvalerate, Bridgeton, N.J., 1985.

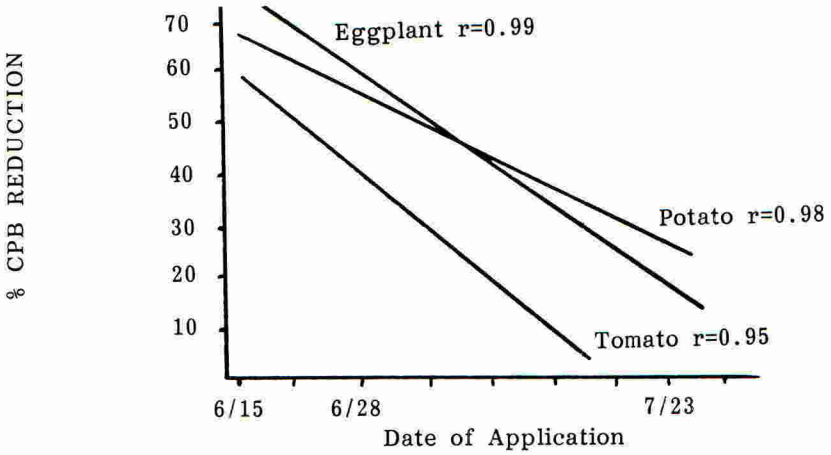

Fig. 3. Percent reduction of Colorado potato beetle after foliar application of fenvalerate (0.0224 kg ai/ha), Bridgeton, N.J., 1984.

These results suggest that sources of host plant resistance other than steroidal glycoalkaloids should be examined. Attempts to increase CPB host-plant resistance by manipulating glycoalkaloids, such as tomatine, or other compounds which require metabolism by pesticide detoxifying enzymes, may increase the potato beetles resistance to insecticides, thereby increasing the pest problem rather than decreasing it. In screening Solanum germplasm for CPB resistance, we identified several clones of S. chacoense which possess field and in vitro resistance to CPB (Carter et al., 1986) and examined some of these for effects on CPB larval response to fenvalerate. Percent mortality following fenvalerate treatment was significantly greater on S. tuberosum cv. 'Katahdin' than on one resistant clone (34% vs. 20%), but addition of piperonyl butoxide overcame this difference, suggesting that increased mixed function oxydase activity was responsible for the greater survival rate of CPB on the resistant clone. Sinden et al. (1986) reported that low levels of a certain class of glycoalkaloids, the leptines, can increase host plant resistance. Leptines are not found in the cultivated potato, but occur to varying degrees in some wild species, e.g, S. chacoense. Significantly, leptines are effective at low concentrations and have not been found in the tubers of resistant S. chacoense clones. This would confer an advantage on breeding for leptine-based resistance. However, CPB adjustment to foliar leptine concentrations may also contribute to enhanced pesticide detoxication ability of CPB. Unless insect resistance is complete, which is rarely the result of breeding programs, some pesticide application may still be necessary. Identification of pesticides whose detoxification is not induced or selected for by feeding on the host plant would, however, alleviate some of the potential for pest control problems with resistant host plants.

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