SESSION 7C

CHEMICAL AND BIOLOGICAL AGENTS FOR PEST AND DISEASE CONTROL: MODES OF ACTION AND METABOLISM

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THE BIOTRANSFORMATION OF 4-CYANO-N,N-DIMETHYLANILINE IN EXCISED SOYA BEAN PLANTS AND IN CALLUS TISSUE CULTURE

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ABSTRACT

Metabolites are often difficult to isolate and identify when pesticides are applied to plants at realistic doses under field conditions. Improved yields can sometimes be obtained using experimental systems under laboratory conditions. The metabolism of a model xenobiotic 4-cyano-N,N-dimethylaniline has been examined in two plant preparations; excised soya bean shoots and plant cell cultures. The major metabolites were isolated and identified by co-chromatography with authentic standards and/or mass spectrometry. The biotransformation pathway in both plant preparations was the same and involved successive N-demethylation followed by sugar conjugation of the mono-methyl and aniline metabolites. However, dramatic quantitative differences were observed in that the cell culture system was apparently deficient in its ability to conjugate the products of phase I metabolism, whereas the excised shoots produced two N-glucosides as the major metabolites.

INTRODUCTION

When pesticides are applied to plants at realistic dose rates under field conditions, metabolites are often difficult to isolate and identify. This is primarily because there are small amounts of metabolites in the presence of large amounts of plant material. To improve the ratio of metabolite to co-extracted plant constitutents, alternative plant preparations can be used e.g. excised leaf discs, excised shoots, callus tissue cultures. These approaches increase the uptake of xenobiotics by exposing a greater surface area of tissue.

This study was designed to investigate the metabolic fate of a model xenobiotic using two plant preparations. Its metabolism in animals has been extensively studied[1,2] and so the work was of interest from a comparative (animal/plant) viewpoint as well as for the development of methods in plant metabolism studies, although only the latter will be discussed here.

MATERIALS AND METHODS

Radiochemical

[^{14}C]4-Cyano-N-N-dimethylaniline (CDA), uniformly labelled in the aromatic ring was used with a specific activity of 9.9 μCi mg^{-1} for the tissue culture experiment and 4.4 μCi mg^{-1} for the excised shoot experiment. The radiochemical purity was >98% by thin layer chromatography in two systems.

Unlabelled Reference Compounds

CDA was obtained from Dr. J. Ashby (Central Toxicology Laboratory, ICI); 4-cyanoaniline (CA), 4-aminobenzoic acid, 4-(methylamino) benzoic acid, 4-dimethylamino benzoic acid were obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset. N-Methyl-4-cyanoaniline (CMA) was synthesised at Sittingbourne Research Centre by partial methylation of 4-cyanoaniline using methyliodide.

Soya bean callus cultures

Soya bean callus (cv. Acme) initiated from cotyledons, was maintained on solid agar medium in the dark at 27°C and for the experiment approximately 0.5 g was sub-cultured into 250 ml conical flasks containing 50 ml liquid medium. The flasks were placed in an orbital incubator at 27°C which breaks down the callus lumps to single cells or small aggregates and aerates the liquid. Ten days after subculture, each flask was treated with 1 mg [^{14}C]CDA dissolved in acetone (50 µl). In addition, one flask containing medium but no cells was treated with [^{14}C]CDA in the same way. The recipe for the nutrient medium was modified from Miller (3).

Soya bean shoots

The stems of soya bean plants (cv. Wells) at the 2 leaf stage were cut under water and the shoots were placed in 20 ml glass scintillation vials (4 plants per vial) containing 10 ml of water/acetone (95:5 v/v), [^{14}C]CDA (352 µg) and sucrose (2.5 µM).

Sampling and Extraction

One tissue culture flask was sampled each day for seven days. The supernatant was removed from the suspended cells by centrifugation followed by filtration and the cells were extracted with ethanol. The radioactivity present in the supernatant and cell extract was determined by liquid scintillation counting (LSC). The unextracted radioactivity in the cells was measured by combustion (Packard B306).

Excised shoots were sampled for extraction every 24 hours for three days. They were homogenised in water/acetone 1:1 (v/v) and the mixture was filtered. The radioactivity in the extract was measured by LSC and the unextracted radioactivity by combustion.

Thin layer chromatography (TLC)

Merck 0.25 mm silica gel F_{254} plates were used with the following solvent systems. (1) Hexane/acetone (7:3), (2) Toluene/ethyl acetate/-acetic acid (75:25:1), (3) Ethyl acetate/ethanol/water (4:2:0.25), all quantities given as volume:volume. Radioactive compounds were located and where necessary quantified by linear analyser (Lablogic Sheffield). Non-radioactive compounds were visualised by viewing the plates at 254 nm.

High performance liquid chromatography (HPLC)

HPLC with UV and radiodetection in series was carried out in 3 systems:

a. Spherisorb ODS (5 µm) 250 x 4.9 mm column; flow rate 1.0 ml min^{-1}; mobile phase A = H_2O, B = CH_3CN, Gradient elution; 0-2 min 100% A, 2-15 min 0% to 15% B in A.

b. as system a. except 250 x 8 mm column, flow rate 2.0 ml min^{-1}; 0-5 min 100% A, 5-25 min 0% to 15% B in A.

c. Partisil SAX (10 µm) 250 x 8 mm column; flow rate 2.0 ml min^{-1}; mobile phase A = H_2O, B = $H_2O/CH_3CN/HCOOH$, 80:10:1 by vol; isocratic elution 5% B in A.

Mass Spectrometry (MS)

Mass spectrometry was carried out on a Finnigin series 4500 in electron impact (EI) and chemical ionisation (CI) modes. Ammonia was the reagent gas for the CI work. Fast Atom Bombardment MS (FAB-MS) was carried out on the polar metabolites with an MS50 medium resolution mass spectrometer.

RESULTS

Metabolism of [^{14}C]4-cyano-N,N-dimethylaniline in soya bean tissue culture

Distribution of radioactivity in cells and supernatant

The total radioactive residues in the supernatant, cell extract and extracted cells following treatment with [^{14}C]CDA were measured at each sampling interval. Most of the radioactivity was found in the supernatant (97-98%) with very little either extractable or bound in the cells. The metabolite profile of the supernatant at each sampling interval was examined by radio-TLC (system 2). The results are summarised in Table 1.

TABLE 1

Percentage composition of supernatant of soya bean tissue cultures following treatment with [^{14}C]CDA

Time (hours)	Metabolite zones (%)			
	A	B	C	D
24	-a	3.3	68.0	28.7
48	0.9	4.9	82.0	12.2
72	1.2	9.7	85.1	4.0
96	1.5	15.2	83.3	-
168	6.6	23.5	69.9	-

A - Rf = 0
B - 4-cyanoaniline
C - 4-cyano-N-methylaniline
D - 4-cyano-N,N-dimethylaniline

a. not detected

The metabolism was rapid with only 4% of the applied radiochemical (D) remaining 72 hours after treatment. The two major metabolites B and C (mono- and di-demethylated CDA) were rapidly formed and released into the medium. Metabolite zone A (Rf = 0), probably a mixture of conjugates, was more slowly formed and was first detected in the medium 48 hours after treatment. From the changing metabolite profile in the supernatant and from structural considerations, it can be concluded that CDA, following rapid uptake, is demethylated to C (4-cyano-N-methylaniline) which is in turn demethylated to B (4-cyanoaniline). Metabolite C is rapidly released in high yield into the medium: 68% of applied radioactivity 24 hours after treatment. Metabolite B is formed more slowly reaching a maximum level of 23% of the applied radioactivity 72 hours after treatment.

Surprisingly, only small amounts of conjugated metabolites were formed. This contrasts with the results from excised soya bean shoots and will be discussed further. The control flask which was analysed 18 days after treatment contained only [^{14}C]CDA.

Identification of metabolites

Metabolites were identified by co-chromatography with standards and their identities were confirmed by mass spectrometry. Isolation was achieved by partitioning the metabolites into ethyl acetate, followed by by preparative thin-layer chromatography (system 2). The metabolite zones were located by radioscanning and were scraped off the plate. The silica gel was eluted with ethyl acetate and the fractions were examined by radio-HPLC (system a) before mass spectrometry.

Metabolite B was identified as 4-cyanoaniline by co-chromatography with the reference standard on TLC (system 2 Rf = 0.4) and HPLC (system a, retention time (Rt) 19 minutes and system c, Rt 6.4 minutes). The CI mass spectrum of the purified metabolite gave peaks at m/z 136($M+NH_4$) and 153($M+N_2H_7$) indicating a molecular weight of 118. The identification was confirmed by electron impact (EI) mass spectrometry which gave a molecular ion M^+ at m/z 118 and loss of HCN to yield a major fragment peak at m/z 91.

Metabolite C when purified co-chromatographed with 4-cyano-N-methylaniline on TLC system 2 (Rf = 0.5) and HPLC system a, (Rt 22 minutes) and c (Rt 8 minutes). The CI mass spectrum gave peaks at m/z 133, 150 and 167 confirming the molecular weight as 132.

Metabolite D co-chromatographed with the starting material (4-cyano-N,N-dimethylaniline) on TLC (system 2 Rf = 0.7) and on HPLC system a (Rt 26 minutes) and system c (Rt 11 minutes). This was confirmed by CIMS which gave peaks at m/z 147, 164 and 181 consistent with a molecular weight of 146.

Metabolism of [^{14}C]-4-cyano-N,N-dimethylaniline in excised soya bean shoots

Distribution of radioactivity

[^{14}C]CDA was taken up rapidly by excised soya bean shoots over three days. At each sampling time the shoots were extracted and the total radioactive residues in the extract and in the extracted plant material were measured. The radioactivity remaining in solution in the glass vials was measured and all these results are illustrated in Figure 1.

At each time point the metabolite profile was determined by TLC solvent system 2. The four metabolite zones were quantified at each sampling point (Table 2). There was a rapid decline of CDA and a concomitant rise in metabolites. In contrast to the tissue cultures these were predominantly polar metabolites.

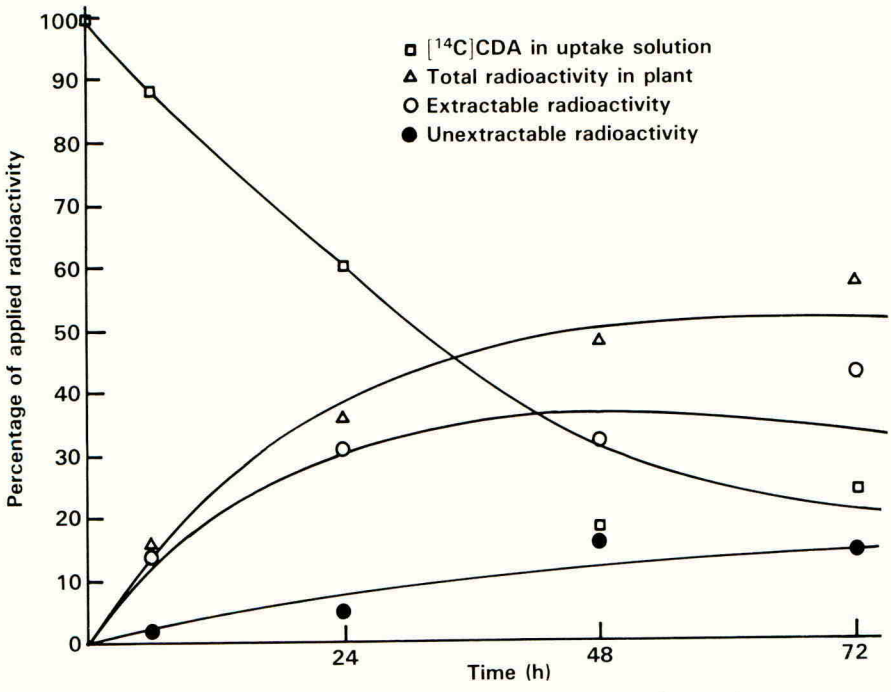

Fig 1 Distribution of radioactivity in excised soya bean shoots treated with [^{14}C]CDA by uptake from aqueous solution

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TABLE 2

Percentage composition of the extractable radioactivity from excised soya bean shoots following treatment with [^{14}C]CDA

Time (hours)	Metabolite zones (%)			
	1	2	3	4
6	1.3	-	6.4	92.3
24	24.8	2.6	23.4	49.2
48	50.6	2.9	16.9	29.6
72	83.5	1.9	7.0	7.6

Metabolite zone
1 N-glucosides
2 4-Cyanoaniline
3 4-Cyano-N-methylaniline
4 4-Cyano-N,N-dimethylaniline

Isolation and identification of metabolites

After extraction the concentrated aqueous solution was partitioned with ethyl acetate. This partition removed metabolites 2, 3 and 4 into the organic phase leaving metabolite 1 in the aqueous phase. Metabolites 2, 3 and 4 were found to have the same chromatographic behaviour as B, C and D on TLC solvent system 2 and HPLC system a. Confirmation that 2, 3 and 4 were 4-cyanoaniline, 4-cyano-N-methylaniline and 4- cyano-N,N-dimethylaniline was obtained by 2 dimensional TLC using solvent systems 1 and 2 and over-spotting with reference standards.

Investigation of the aqueous phase by HPLC system b showed two radioactive components at retention times 21 minutes and 22 minutes. These were designated metabolites 1a and 1b respectively and were present in the ratio 1:2 approximately. Primary clean-up of the aqueous phase was carried out by TLC, solvent system 3. The radioactivity was located by autoradiography, scraped from the plate, and eluted with methanol. The metabolites were separated and cleaned up by repeated injections on HPLC system b.

Metabolite 1a was the more polar of the two metabolites. A sample, isolated as described above was submitted for FAB mass spectrometry in methanol. The FAB mass spectrum gave a weak $[M-H]^-$ ion at m/z 279 which corresponds to a molecular formula of $C_{13}H_{16}N_2O_5$. This is consistent with the metabolite being 4-cyanoaniline with a C_6 sugar attached. Metabolic studies with other xenobiotics in plants indicate that the conjugating sugar moiety is normally glucose[3]. The evidence is consistent with metabolite 1a being the N-glucoside of 4-cyanoaniline.

Metabolite 1b was the less polar of the two metabolites. A sample was isolated as described above and dissolved in methanol for FAB mass spectrometry. The FAB negative ion mass spectrum gave $[M-H]^-$ at m/z 293 with its glycerol adduct at m/z 385, and an intense ion at m/z 131 which could be due to 4-cyano-N-methylaniline moiety. Acid hydrolysis of this metabolite released a product with an Rf 0.5 on TLC solvent system 2. This area was scraped from the plate, eluted with ethyl acetate and submitted for CI mass spectroscopy. The CI mass spectrum confirmed the identity of product as 4-cyano-N-methylaniline. This evidence is consistent with metabolite 1b being the N-glucoside of 4-cyano-N-methylaniline.

DISCUSSION

Plant tissue culture and excised leaf techniques are useful for studying the metabolism of xenobiotics in plants. With both systems uptake and metabolism of [^{14}C]CDA was rapid; generous quantities of metabolites were isolated for identification.

Although the metabolite profiles were qualitatively similar for cultures and excised shoots i.e. the same metabolic pathway was apparently operating in both systems (Figure 2) there were large quantitative differences. The excised shoot preparation rapidly conjugated the demethylated metabolites so that 72 hours after treatment over 80% of the extractable radioactivity was present as sugar conjugates. In the tissue culture preparation polar metabolites were much less rapidly formed so that 72 hours after treatment only 2% of the recovered radioactivity was present as polar metabolites. The products of N-demethylation were rapidly released into the medium where over 97% of the radioactivity was recovered at each sampling interval. The polar metabolites comprised a larger proportion of the intracellular radioactivity (approximately 30%) but in overall terms this was insignificant.

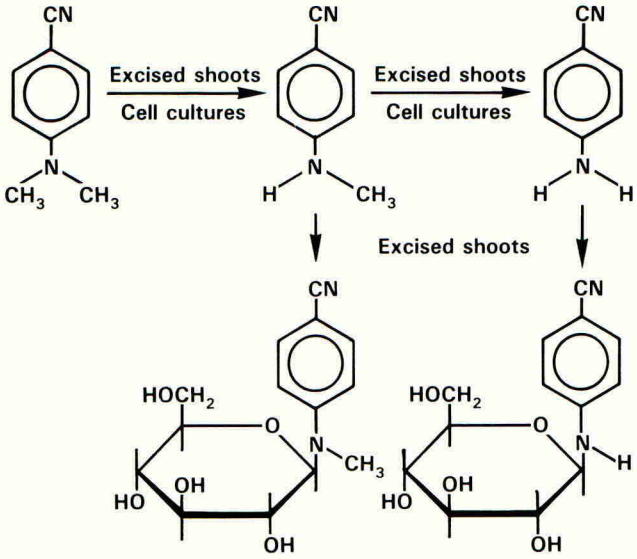

Fig 2 The biotransformation of [^{14}C]CDA excised in soya bean shoots and soya bean callus cultures

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It was surprising to find this lack of conjugation in the plant tissue culture system. The metabolism of 4-chloro-2-methyl phenoxy acetic acid (MCPA) was studied in wheat callus culture and excised wheat shoots[4]. In both cases the major metabolite was an ether-linked sugar conjugate of a hydroxylated metabolite. There are many examples of sugar conjugation of pesticides in plant tissue culture[5,6]. The inefficiency of the callus tissue in N-glucosylation was probably due to the loss of substrate to the culture medium, rather than to a metabolic deficiency. There was no evidence for other potential metabolic pathways e.g. the formation of N-hydroxymethyl glucosides, aromatic hydroxylation or metabolism of the nitrile group in either preparation.

Thus although the tissue culture and excised shoots produced quantitatively different results, the two techniques complemented one another. The tissue culture preparations were used for the identification of the free metabolites which were also the aglycones of the conjugated metabolites. With this prior knowledge, the isolation and identification of the N-glucosides from the intact leaf preparation was facilitated.

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UPTAKE AND METABOLISM OF TEFLUTHRIN BY MAIZE AND SUGAR BEET

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ABSTRACT

The uptake and metabolism of ^{14}C-tefluthrin ('Force') by maize and sugar beet plants was studied. Plants were grown in soil treated with a granular application of ^{14}C-cyclopropane-labelled and ^{14}C-phenyl-labelled tefluthrin. Radioactive residues were found in the foliage of all crops (0.01-0.46 mg/kg) and the sugar beet root (0.04 mg/kg). Very low residues (<0.01 mg/kg) were detected in the maize grain. The residues were identified mainly as free and conjugated metabolites of tefluthrin. Only a small part of the residue was due to tefluthrin itself.

The metabolism of ^{14}C-tefluthrin was also studied after a granular application was made to maize whorls. At harvest, radioactive residues were detected in the foliage (0.55-1.21 mg/kg) but only very low levels were detected in the grain (<0.01 mg/kg). Tefluthrin was detected at moderate levels in the foliage (~0.19 mg/kg) but most of the residue was due to metabolites of tefluthrin.

INTRODUCTION

Tefluthrin, (2,3,5,6-tetrafluoro-4-methylbenzyl cis-3-(Z-2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate or 2,3,5,6-tetrafluoro-4-methylbenzyl (Z)-(1R,3R-;1S,3S)-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropane-carboxylate) is a novel pyrethroid soil insecticide for use mainly in maize and sugar beet. It shows excellent efficacy against US corn rootworm (Diabrotica sp.). It also performs well as a whorl treatment in maize against European corn borer (Ostrinia (Pyrausta) nubilalis), and shows activity against US cutworm (Agrotis ipsilon), white grubs (Melolontha melonontha), black maize beetles (Heteronychus spp), earwigs (Forficula cuvicularia), symphilids (Scutigerella sp.) and millipedes (Blaniulus guttulatus, Brachydesmus superus) (Jutsum et al. 1986).

It is currently formulated as a 0.5-1.5% gypsum granule. Maize and sugar beet have been grown in soil treated with a granular formulation of ^{14}C-tefluthrin, and its uptake and degradation has been investigated.

MATERIALS AND METHODS

Radiochemicals

Two batches of ^{14}C-tefluthrin were synthesised by the Radiochemical Synthetic Unit, ICI Plant Protection Division, Jealott's Hill Research Station. The first was radiolabelled in the 2,2-dimethylcyclopropane ring (^{14}C-cyclopropane-labelled tefluthrin) and the second in the tetrafluoromethylbenzyl ring (^{14}C-phenyl-labelled tefluthrin), as shown in Fig. 1.

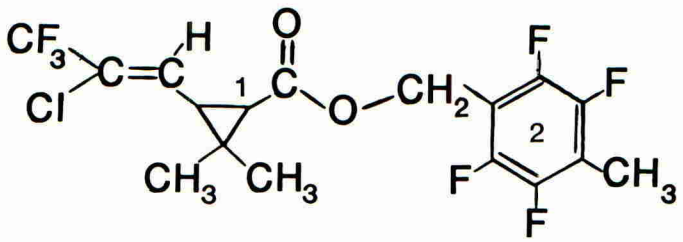

Fig. 1. Positions of radiolabelling in ^{14}C-tefluthrin

1. ^{14}C-cyclopropane-labelled tefluthrin, (specific activity 0.87 MBq/mg)
2. ^{14}C-phenyl-labelled tefluthrin, (specific activity 0.83 MBq/mg) N.B. Bq = Bequerel

The radiochemicals were formulated as 1.4% gypsum granules which were then either applied to soil in which maize and sugar beet were to be grown, or to the maize whorls, 3-4 weeks after germination.

Experiment 1

23cm and 32cm diameter pots were nearly-filled with a sandy loam soil, which was then treated at a rate equivalent to 0.8 kg a.i./ha with either ^{14}C-cyclopropane- or ^{14}C-phenyl-labelled tefluthrin-treated granules. The granules were then covered with 5mm of soil.

Maize (v. "Northern Star") and sugar beet (v. "Amono") were planted immediately after treatment of the soil (maize 23cm pots, sugar beet 32cm pots), and grown in a glasshouse at temperatures ranging from 14-50°C. All crops were harvested between 76 and 82 days after treatment of the soil.

Experiment 2

23cm pots were filled with the sandy loam soil and maize (v. "Northern Star") was planted. 21 days after germination the maize whorls were treated with the granular formulation of ^{14}C-tefluthrin, at a rate equivalent to 0.3 kg a.i./ha. The crop was grown as in experiment 1. The maize was harvested between 55 and 61 days after treatment.

Extraction of radioactive residues from crops

Generally, radioactive residues were extracted from maize and sugar beet tissues by homogenising with acetonitrile and/or acetonitrile/distilled water (1:1). Any residues remaining bound to the plant matrix were extracted by refluxing with hydrochloric acid (1M, 2M or 6M). Extracts were centrifuged and aliquots (0.2-2 ml) were assayed by liquid scintillation counting (lsc). The plant debris remaining after extraction was thoroughly dried and combusted (Harvey OX300 Biological Oxidiser) prior to lsc to determine the level of unextracted activity.

Characterisation of residues in crops

Most extracts were analysed directly by normal phase thin layer chromatography (tlc, Machery-Nagel pre-coated silica plates 0.25mm). Following this, acetonitrile extracts were concentrated by rotary evaporation to a small volume and partitioned between water and an organic solvent (eg hexane, dichloromethane, or ethyl acetate). The radioactivity remaining in the water-soluble fraction was then hydrolysed in 2M hydrochloric acid (reflux, 2 h) and occasionally in 6M hydrochloric acid, (reflux 6 h). After hydrolysis, the radioactivity was again partitioned between acid and an organic solvent. The organic fractions generated by hydrolysis were analysed by tlc. The solvent systems used in tlc were as follows:

Solvent system 1: hexane/diethyl ether 25:1 v/v
Solvent system 2: hexane saturated with acetonitrile
Solvent system 3: cyclohexane saturated with formic acid/diethyl ether 3:2 v/v
Solvent system 4: chloroform/acetonitrile/formic acid 90:10:0.1 v/v/v
Solvent system 5: hexane/ethyl acetate/methanol/formic acid 70:30:3:3 v/v/v/v

Gas chromatography/mass spectrometry (gc/ms) was carried out using a Finnigan Mat 8200 mass spectrometer and a Varian 3700 capillary gas chromatograph, linked to a Finnigan Incos Data System.

RESULTS

Experiment 1, soil treatment

Maize plants were separated into foliage, stem and grain, and sugar beet were separated into foliage and root.

Radioactive residues had translocated into crops grown in both the ^{14}C-cyclopropane- and ^{14}C-phenyl-labelled tefluthrin treated soils. These residues ranged from <0.01 mg/kg (maize grain) to 0.09 mg/kg (sugar beet foliage) in the ^{14}C-cyclopropane-labelled tefluthrin experiment, and from <0.01 mg/kg (maize grain) to 0.46 mg/kg (maize leaves) in the ^{14}C-phenyl-labelled tefluthrin experiment.

Residue levels in all crops are summarised in Table 1 below.

TABLE 1

^{14}C-Residues in mature crops

Radiolabel	Crop	Residue, mg/kg
Cyclopropane	Maize leaves	0.06
	Maize stem	0.01
	Maize grain	<0.01
	Sugar beet foliage	0.09
	Sugar beet root	0.04
Phenyl	Maize leaves	0.46
	Maize stem	0.08
	Maize grain	<0.01
	Sugar beet foliage	0.30
	Sugar beet root	0.04

Characterisation of residues

^{14}C-cyclopropane-labelled tefluthrin

Radioactive residues were characterised in maize leaves, sugar beet foliage and sugar beet root by co-chromatography (tlc) with authentic reference compounds. Chromatograms were run in solvent systems 3 and 4.

Tefluthrin itself was only detected at very low levels in the crops (<0.01 mg/kg). The two major components of the residues were conjugated (1RS)-cis-3-(Z-2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethyl-cyclopropanecarboxylic acid (Compound II) and conjugated 3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2-hydroxymethyl-2-methylcyclopropanecarboxylic acid (Compound III). Table 2 shows the percentages of these compounds detected in the crops. The figures are calculated as percentages of the overall residues detected.

TABLE 2

Compounds detected in crops (^{14}C-cyclopropane-labelled tefluthrin, soil treatment)

Crop	tefluthrin	Compound II	Compound III	Water-soluble	Unknowns
Maize leaves	7%	15%	18%	24%	36%
Sugar beet foliage	-	59%	7%	20%	15%
Sugar beet root	5%	77%	6%	7%	5%

^{14}C-phenyl-labelled tefluthrin

Radioactive residues were characterised in the maize leaves, maize stem, sugar beet foliage and sugar beet root by tlc. Chromatograms were run in solvent systems 5 and 6.

Tefluthrin was only detected at very low levels in the crops (<0.01 mg/kg). The major component of the residues was free and conjugated 2,3,5,6-tetrafluoro-4-hydroxymethylbenzoic acid (Compound IV). Very low levels of 2,3,5,6-tetrafluoro-1,4-benzenedicarboxylic acid (Compound V) were also detected in the maize leaves. Table 3 shows the percentages of these compounds detected in the crops.

TABLE 3

Compounds detected in the crops (^{14}C-phenyl-labelled tefluthrin, soil treatment)

Crop	tefluthrin	Compound IV	Compound V	Water-soluble	Unknowns
Maize leaves	<1%	77%	5%	5%	13%
Maize stem	-	84%	-	7%	9%
Sugar beet foliage	2%	96%	-	1%	1%
Sugar beet root	-	42%	-	50%	8%

Mass spectral confirmation of compound IV as the major metabolite in sugar beet foliage

Compound IV was isolated from the sugar beet foliage extracts by reverse phase high performance liquid chromatography (hplc, Waters Assoc.). The isolate was concentrated to dryness, redissolved in dry ethyl acetate (1 ml) and mixed with ethereal diazomethane (~2 ml). The mixture was allowed to stand (1 h) before evaporating to dryness and redissolving in dry acetonitrile (0.3 ml). N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA, Pierce Chemical Co., 0.5 ml) was added and the reagents were heated (70°C, 1 h). The resulting solution was evaporated to dryness and then purified by normal phase hplc, to yield methyl 2,3,5,6-tetrafluoro-4-trimethylsilyloxymethylbenzoate.

Mass spectral analysis (electron impact, following gas chromatography) of the isolate and comparison with an authentic compound confirmed the structure of this compound: (m/z 310, 1%, M^+; m/z 295, 100%, M^+-CH_3; m/z 279, 25%, M^+-CH_3O; m/z 265, 23%, $[M^+-CH_3]-CH_2O$; m/z 187, 70% structure not assigned.)

Experiment 2 whorl treatment

Radioactive residues did not translocate through the maize plants to the seeds but remained in the leaves for both the ^{14}C-cyclopropane- and ^{14}C-phenyl-labelled tefluthrin treatments. Residues ranged from <0.01 mg/kg (grain) to 1.21 mg/kg (leaves) in the ^{14}C-cyclopropane-labelled tefluthrin treatment, and from <0.01 mg/kg (grain) to 0.55 mg/kg (leaves) in the ^{14}C-phenyl-labelled tefluthrin treatment.

Residue levels in both crops are summarised in Table 4 below.

TABLE 4

Residue levels in mature crops

Radiolabel	Crop part	Residue, mg/kg
Cyclopropane	Leaves	1.21
	Stem	0.05
	Grain	<0.01
Phenyl	Leaves	0.55
	Stem	0.01
	Grain	<0.01

Characterisation of residues

^{14}C-cyclopropane-labelled tefluthrin

Radioactive residues were characterised in leaves and stem by tlc in solvent systems 1,2,3 and 4. Tefluthrin was detected in both leaves and stem, together with conjugated (1RS)-cis-3-(Z-2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylic acid (Compound II) and conjugated 3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2-hydroxymethylcyclopropanecarboxylic acid (Compound III). Low levels of conjugated (1RS-trans-3-(ZE-2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylic acid (Compound VI) and conjugated 2,3,5,6-tetrafluoro-4-hydroxymethylbenzyl cis-3-(Z-2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate (Compound VII) were also detected.

Table 5 shows the percentages of these compounds detected in the crops.

TABLE 5

Compounds detected in crops (^{14}C-cyclopropane-labelled tefluthrin, whorl treatment)

Crop part	tefluthrin	Compound II	Compound III	Compound VI	Compound VII
Leaves	8%	45%	20%	3%	<1.0%
Stem	60%	13%	2%	-	-

The remaining radioactivity was water-soluble (12-11%) or unidentified (13-14%).

^{14}C-phenyl-labelled tefluthrin

Radioactive residues were characterised in the leaves by tlc in solvent systems 1,2,5 and 6. Tefluthrin and low levels of its trans isomer were detected. The major component of the residue was conjugated 2,3,5,6-tetra-fluoro-p-xylene-α,α'-diol (Compound VIII). Low levels of conjugated 2,3,5,6-tetrafluoro-4-methylbenzyl alcohol (Compound IX) and free and conjugated 2,3,5,6-tetrafluoro-4-hydroxymethylbenzyl cis-3-(Z-2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate (Compound VII) were also detected. Table 6 shows percentages of the compounds detected in the crops.

TABLE 6

Compounds detected in crops (^{14}C-phenyl-labelled tefluthrin, whorl treatment)

Crop part	tefluthrin	trans-tefluthrin	Compound VII	Compound VIII	Compound IX
Leaves	16%	3%	47%	10%	10%

The remaining radioactivity was water-soluble (2%) or unidentified (13%).

DISCUSSION

Maize and sugar beet plants were grown in soil treated with granular formulations of ^{14}C-cyclopropane- and ^{14}C-phenyl-labelled tefluthrin. Radioactivity translocated into the foliage of both crops, but no residues (<0.01 mg/kg) were detected in the maize grain and only low residues (0.04 mg/kg) were present in the sugar beet root. Most of the residue in the crops was due to metabolites of tefluthrin, with only a small part due to tefluthrin itself.

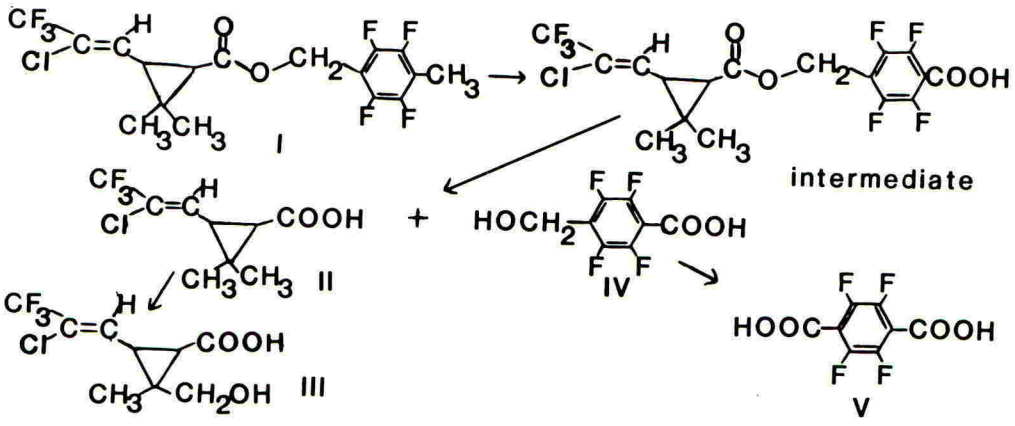

Fig. 2 shows the proposed metabolic pathway for tefluthrin from a soil application.

Maize plants were also treated in the whorls. Radioactive residues remained in the leaves but only very low levels were detected in the grain. Tefluthrin was detected in the foliage at moderate levels (~0.19 mg/kg^{-1}). However, most of the radioactivity in the foliage was due to metabolites of tefluthrin.

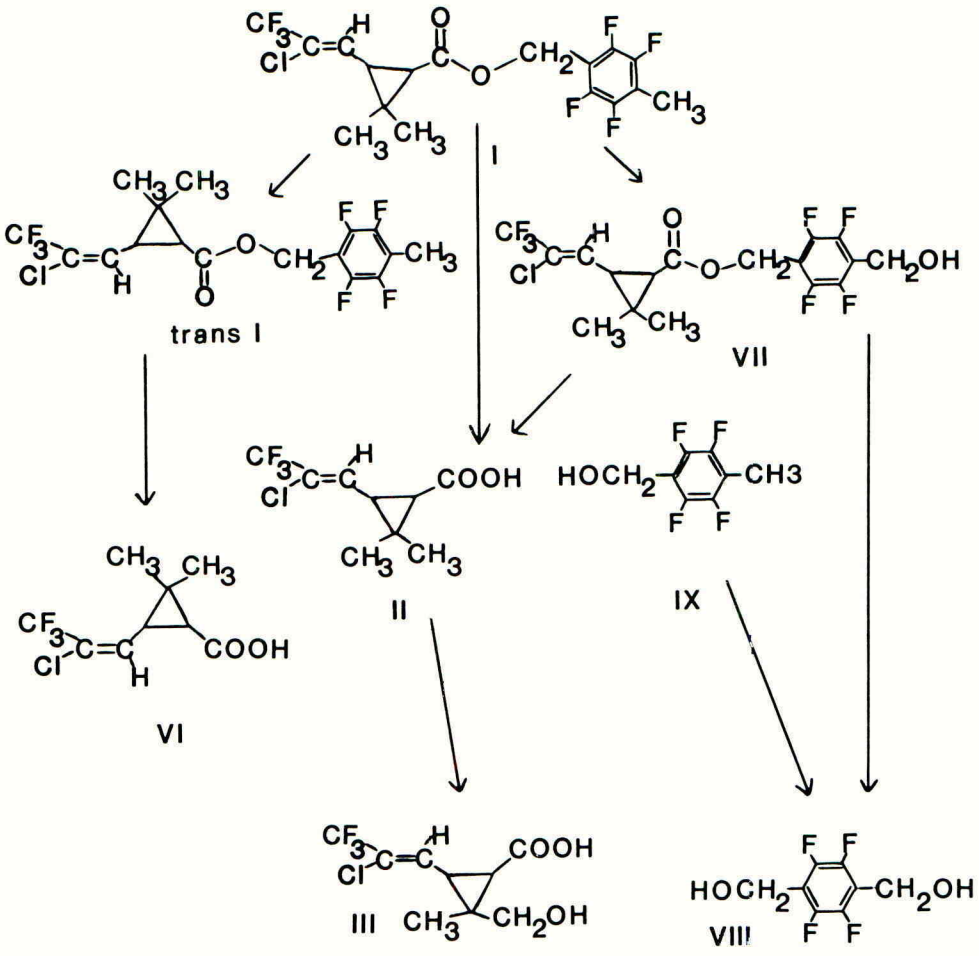

Fig. 3 shows the proposed metabolic pathway for tefluthrin in maize treated in the whorls.

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PLANT COMPOUNDS THAT SYNERGISE ACTIVITY OF THE APHID ALARM PHEROMONE

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ABSTRACT

Several isothiocyanates have been found to synergise the activity of (E)-β-farnesene, the aphid alarm pheromone, against the mustard aphid (*Lipaphis erysimi*). While there was no clear indication of the existence of such synergists of the alarm pheromone for *Myzus persicae*, there was evidence that *Aphis fabae* employs alarm pheromone synergists derived from its host plant.

INTRODUCTION

The alarm pheromone of most aphid species is (E)-β-farnesene (Bowers *et al.* 1972, Pickett & Griffiths, 1980). It is released when an aphid is attacked by parasites or predators and causes neighbouring aphids to cease feeding and to move rapidly away.

Nault & Bowers (1974) demonstrated that the turnip aphid, *Lipaphis (Hyadaphis) erysimi* produced (E)-β-farnesene as an alarm pheromone component but did not respond well to this compound alone. Dawson *et al.* (1987) have since shown that plant-derived synergists must also be present for full alarm response by this species (see Table 1). The synergists identified included allyl, 2-butyl, 3-butenyl and 4-pentenyl isothiocyanates. Olfactory receptors responding to the synergists were located on the rhinarium of the fifth antennal segment, whereas (E)-β-farnesene was detected by the rhinarium on the sixth segment.

The increase in aphid movement caused by the alarm pheromone can improve pick-up of insecticide and the effectiveness of insecticide-pheromone combinations has been described (Griffiths & Pickett, 1980). However, some economically important aphid species do not respond well to currently available alarm pheromone formulations. Thus the discovery of plant-derived alarm pheromone synergists for *L. erysimi* has led us to investigate whether such compounds also exist for the peach potato aphid, *Myzus persicae* and the black bean aphid, *Aphis fabae*.

MATERIALS AND METHODS

Compounds

Farnesene, prepared by the method of Dawson *et al.* (1982), containing 40% of the active isomer (E)-β-farnesene (EBF), was employed at a concentration of 0.4 mg/ml EBF in hexane, or 1 μg/ml in water, and stored in glass ampoules under N_2. Allyl, 2-butyl and 3-butenyl isothiocyanates were obtained commercially or synthesised by conventional methods and employed at 0.02 mg/ml in hexane or 1 mg/ml in water.

Insects

All the aphids used in our tests were from strains that were

maintained parthenogenetically at 16 h daylength and 20 ± 5°C. *M. persicae* and *A. fabae* were insecticide-susceptible laboratory strains. *L. erysimi* was obtained from Shepherd's purse (*Capsella bursa-pastoris*) in 1981. *M. persicae* and *L. erysimi* were maintained on Chinese cabbage (*Brassica campestris* var. *chinensis* (L.) Makino) and *A. fabae* on bean (*Vicia faba*).

Isolation of volatiles

A. fabae were vacuum distilled as described previously (Pickett and Griffiths, 1980). The concentration of the aqueous distillate was approximately 1 g aphid/1 ml distillate.

Aerial parts of bean and Chinese cabbage plants grown under glass were cooled under nitrogen, broken up finely, covered and allowed to warm up to room temperature. The volatiles were then isolated by condensation as described previously (Pickett and Stephenson, 1980). The concentration of the distillate was approximately 1 g plant material/1 ml distillate. The aqueous distillates were sealed in glass ampoules under nitrogen and stored at -20°C.

Bioassays

There was evidence from preliminary tests that aphids in colonies are more responsive to alarm pheromone than aphids reared singly. As the bioassays were designed to detect improvements in the response to pheromone, tests were performed using single aphids of the more responsive species (*L. erysimi* and *M. persicae*) and colonies of the less responsive *A. fabae*. With the latter aphid it was also necessary to avoid the use of organic solvents (Dawson *et al.*, 1982).

Aphid colonies were established on leaves of host plants by confining adults in clip cages on the plants for 3 days. The adults were then removed and the young allowed to develop for a further 2-3 days before testing. Single aphids were reared in a similar manner, excess nymphs being removed at the same time as the adults.

Test materials were applied in air (20 ml from 20 crushed aphids, containing a total of ca. 10 ng EBF), blown slowly over test insects during 10 secs from a glass syringe, or placed on the leaf in a drop of solvent (0.2 µl), either hexane (containing 80 ng EBF or 4 ng isothiocyanate) or water (containing 0.2 ng EBF or 200 ng isothiocyanate). Aqueous distillates of aphids or beans were applied as single µl drops.

Electrophysiology

Recordings from the cells associated with the olfactory receptors on the primary rhinaria of alates were made using tungsten microelectrodes (Boeckh, 1962). The indifferent electrode was placed in the first antennal segment and the recording electrode was then brought into contact with the multiporous plate of the rhinarium until impulses were recorded. Permanent copies of the action potentials generated by the receptor cells were obtained by standard methods (Wadhams *et al.*, 1982).

Gas Chromatography coupled Single Cell Recording (GC-SCR)

The coupled GC-SCR system has been described previously (Wadhams, 1982). After each experiment, records of the flame ionisation detector (FID) response and of the action potential frequency were obtained by detecting the impulses with a level discriminator and plotting them by means of a voltage/frequency converter.

RESULTS AND DISCUSSION

Table 1 shows that in the alarm bioassay, *L. erysimi* on Chinese cabbage responded fully to vapour from crushed aphids but responded only poorly to (E)-β-farnesene alone. Addition of host-plant volatiles to the (E)-β-farnesene dramatically increased this response. As described in Dawson *et al.* (1987), GC-SCR techniques were used to locate electrophysiologically active peaks and Gas Chromatography coupled Mass Spectrometry (GC-MS) was used to identify them. One of the most active plant components identified by these means was 3-butenyl isothiocyanate.

TABLE 1

Alarm bioassay with *Lipaphis erysimi* (single aphids)*

Treatment	% aphids moving
Vapour from crushed *Lipaphis erysimi*	100[a]
EBF	20[b]
EBF + Chinese cabbage volatiles	90[a]
EBF + 3-butenyl isothiocyanate	90[a]
Difference [a] from [b], P = < 0.05	

* full data in Dawson *et al.* (1987).

As *M. persicae* also colonises Chinese cabbage, the effects of the host plant volatiles were examined with this species (Table 2). However, neither the total volatiles, nor the individual compounds known to synergise activity against *L. erysimi* were effective in improving the performance of (E)-β-farnesene against single *M. persicae*. Response of single *M. persicae* to crushed aphids was also low. As previously noted (Dawson *et al.*, 1982) colonies of *M. persicae* respond well to (E)-β-farnesene (Table 2) indicating that for this aphid, rearing conditions are the most important factor for good response. Dependence on specific plant compounds would not be expected in a species with such a wide and diverse host range.

TABLE 2

Alarm bioassay with *Myzus persicae*

Treatment		% aphids moving	
a) Single aphids	Vapour from crushed *M. persicae*	34	
	EBF	27	
	EBF + allyl isothiocyanate	50	not sig. diff.
	EBF + 2-butyl isothiocyanate	20	not sig. diff.
	EBF + 3-butenyl isothiocyanate	50	not sig. diff.
	EBF + Chinese cabbage volatiles	50	not sig. diff.
b) Colonies	EBF	93 ± 6.5	

In contrast, colonies of *A. fabae* responded only poorly to (E)-β-farnesene alone (Table 3). Vapour from crushed aphids produced a moderately strong response, indicating that additional components may be important. The isothiocyanates that synergise the response of *L. erysimi* do not improve the activity of (E)-β-farnesene against *A. fabae* (Table 3), and this result is predictable as *A. fabae* does not colonise cruciferous plants of which the isothiocyanates are characteristic components.

An aqueous distillate of *A. fabae* elicited only a weak alarm response, as did the residue from the distillate, and although a combination of the two gave a significantly greater response, it still did not reach the level obtained with freshly crushed aphids. However, to obtain sufficient material for the distillation, the aphids were stored at -20°C for up to a week and it is possible that some autolysis of labile components may have taken place. Since the crushed aphids used in the alarm bioassay were taken directly from the plant, it was considered likely that larger amounts of host plant compounds would have been present. Indeed, it was found that addition of bean volatiles to the distillate/residue mixture restored the alarm response to the level achieved with freshly crushed aphids. Thus it would appear that the alarm pheromone in *A. fabae* is a multicomponent mixture and that, as with *L. erysimi*, synergists derived from the host plant are involved.

TABLE 3

Alarm bioassay with *Aphis fabae* (colonies)

Treatment	% aphids moving ± standard error
Vapour from crushed *Aphis fabae*	77.0 ± 7.48
Aqueous solution of	
EBF	2.1 ± 1.5
EBF + allyl isothiocyanate	0.7 ± 0.42
EBF + 3-butenyl isothiocyanate	0.0
Aphis fabae distillate	17.0 ± 5.90
Aphis fabae residue	25.0 ± 8.92
Aphis fabae distillate + residue	46.0 ± 11.78
Aphis fabae distillate + residue + bean volatiles	73.0 ± 4.49

Electrophysiological recordings from the primary rhinarium on the 5th antennal segment of *A. fabae* have shown the presence of olfactory receptors which respond to plant-derived compounds. Fig. 1 shows the response of a number of cells on the rhinarium to stimulation with a mixture of bean volatiles. GC-SCR of this mixture has revealed the presence of at least 3 electrophysiologically active components in the extract (Fig. 2) and further work is in progress, both to identify these compounds and to determine their role in the chemically mediated behaviour of *A. fabae*.

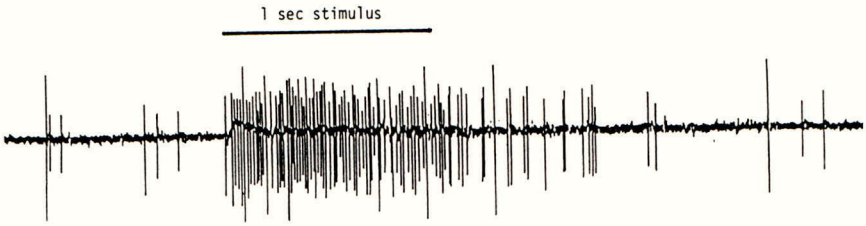

FIG. 1 Response of A. fabae olfactory receptors to bean volatiles

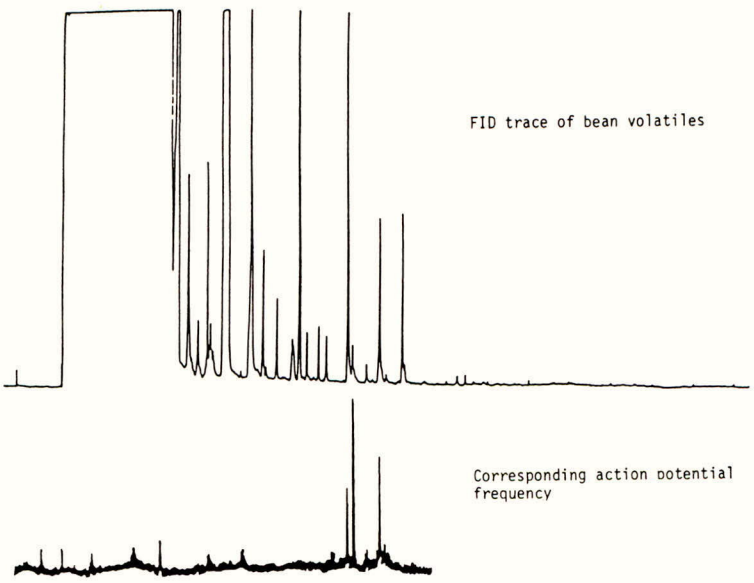

FIG 2 Coupled GC-SCR of a mixture of bean volatiles

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THE STABILITY OF ENANTIOMERS OF TRIADIMEFON AND TRIADIMENOL

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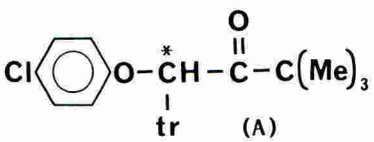
ABSTRACT

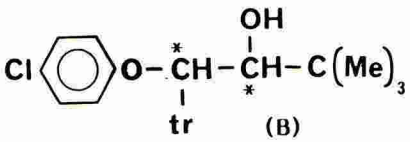
The stability of enantiomers of both triadimefon and triadimenol in aqueous solution buffered at pH values from 4 to 8 was measured. The stability of triadimenol enantiomers when added to cultures of fungi growing in liquid medium was also investigated. In solution, triadimefon underwent base-catalysed racemisation but no comparable changes were observed for triadimenol enantiomers. Enantiomers of triadimenol showed no change of chiral integrity, or oxidation to triadimefon, when incubated with fungal cultures.

INTRODUCTION

The ability of fungi to reduce triadimefon [1-(4-chloro-phenoxy)-3,3-dimethyl-1-(1,2,4-triazol-1-yl)butan-2-one] to triadimenol, the corresponding 2-ol, is well documented (Clark *et al.* 1978; Gasztonyi & Josepovits 1979; Gasztonyi 1981; Deas & Clifford 1982; Buchenauer & Grossman 1982; Deas *et al.* 1984a,b,c; Deas & Clifford 1984; Deas *et al.* 1986). This conversion has been regarded as an 'activation' process (Gasztonyi & Josepovits 1979), triadimefon *per se* being regarded as not fungitoxic. The oxidative 'deactivation' of triadimenol to triadimefon has also been observed when the former was incubated with *Coriolus versicolor* or *Rhizoctonia solani* (Deas & Clifford 1984).

Triadimefon (Fig. 1A) has one chiral centre and thus can exist in two enantiomeric forms, 1R and 1S. Triadimenol (Fig. 1B) possesses two chiral centres and can, therefore, exist in four forms 1R,2S; 1S,2R; 1R,2R and 1S,2S which can be grouped into two pairs that are diastereoisomerically related viz:- 1R,2S; 1S,2R and 1R,2R; 1S,2S.





* = chiral centre
tr = 1,2,4-triazol-1-yl

Fig. 1. The structures of triadimefon and triadimenol.

It has been demonstrated (Krämer *et al.* 1983; Deas *et al.* 1986) that triadimenol enantiomers differ greatly in their fungitoxicity, the 1S,2R form being the most active. Assuming stereochemical stability of both precursor and product, 1R triadimefon can yield only 1R,2R and 1R,2S triadimenols and likewise the 1S triadimefon yields only the 1S,2R and 1S,2S forms. It would be expected, therefore, that the two enantiomers

of triadimefon would show different levels of fungitoxicity, but in practice, they show very similar activity against a wide range of fungi (Krämer et al. 1983; Deas et al. 1986).

Furthermore, when Fusarium culmorum was incubated with a 1 : 1 mixture of 1R and 1S triadimefon enantiomers, complete conversion to triadimenol was observed (Deas et al. 1986). The triadimenol products possessed 1R : 1S-based configurations in the ratio of approximately 1 : 2 compared with a predicted value of 1 : 1.

The above observations suggest that the stereochemical integrity of triadimefon enantiomers was not maintained in the environment prevailing during our fungicide assays and metabolism studies. In preliminary experiments (unpublished) individual triadimefon enantiomers were incubated in liquid nutrient media and aqueous buffers. Under these conditions racemisation of both the 1R and 1S enantiomers occurred to varying extents, apparently related to the pH of the medium.

In the experiments described here, a detailed time-course investigation into the racemisation of triadimefon enantiomers in buffer solutions was undertaken. The stereochemical stability of individual enantiomers of triadimenol was also examined, both in buffer solutions and when added to fungal cultures.

MATERIALS AND METHODS

Chemicals

Chromatographically pure triadimefon had been isolated previously (Clark et al. 1978). Individual 1R and 1S enantiomers of triadimefon, [^{14}C]-labelled in the triazole ring and with specific activity 2.11 x 10^5 Bq/mg, were prepared using published methods (Deas et al. 1986). Unlabelled individual 1R,2S; 1S,2R; 1R,2R and 1S,2S enantiomers of triadimenol [1-(4-chlorophenoxy)-3,3-dimethyl-1-(1,2,4-triazol-1-yl) butan-2-ol] and their [^{14}C]-analogues (U-labelled in the phenyl-ring, 2.46 x 10^6 Bq/mg) were kindly supplied by Bayer A.G.

Fungi

Isolates of the following species were taken from culture collections held at Long Ashton Research Station:- Gaeumannomyces graminis, Botrytis cinerea, Rhizoctonia cerealis, Pyrenophora teres, Fusarium culmorum and Glomerella cingulata.

Stability of enantiomers in buffer solutions

The 1R or 1S enantiomer of [^{14}C]-triadimefon (3.61 x 10^3 Bq/mg; 0.51 mg dissolved in 0.5 ml acetone) was added separately to ten 100 ml Erlenmeyer flasks each containing 50 ml of sterile 0.2M phosphate/citrate buffer (pH 4, 5, 6, 7 or 8). The flasks were incubated on an orbital shaker at 25°C in darkness. At intervals, batches of two flasks per treatment were taken, their contents combined and extracted with diethyl ether (3 x 50 ml). The combined ether extracts were dried over anhydrous sodium sulphate and the solvent removed in a rotary evaporator at 30°C.

Similarly, single enantiomers of [^{14}C]-triadimenol (1.83 x 10^4 Bq/mg; 0.5 mg in 0.5 ml acetone) were added to sterile buffer solutions (pH 4, 6 or 8) and extracted after incubation for 72h.

Stability of enantiomers in fungal cultures

Test fungi were grown in submerged shake culture in a sterile liquid

medium containing glucose (10 g/l) and yeast extract (Oxoid L21, 2 g/l). Batches of twelve 100 ml Erlenmeyer flasks, each containing 50 ml medium, were each inoculated with five agar plugs (5 mm diam.) cut from 4-day old cultures of one of the six test fungi. Inoculated flasks were incubated at 25°C in darkness on an orbital shaker (150 rev/min, 72h). Two flasks of each fungus were filtered and the pH of the filtrate and the dry weight of mycelium present were determined. Individual, unlabelled enantiomers of triadimenol (0.4 mg in 1 ml acetone) were each added to two flasks of each fungus, the remaining two flasks receiving 1 ml acetone only. All flasks were returned to the shaker for a further 48h after which they were filtered and the pH of the filtrate and dry weight of the mycelium were determined. Filtrates from the duplicate flasks were combined and extracted with diethyl ether as described above.

Analysis of extracts

Determination of [^{14}C] content

The total [^{14}C]-content of the extracts was determined by liquid scintillation counting using an LKB Rackbeta instrument and LKB Optiphase RIA scintillant.

Triadimefon enantiomer content

Hplc analyses were conducted using a Waters Associates 6000A pump, U6K injector and model 440 U.V. detector (280 nm). The chiral column used (30 cm x 0.8 cm i.d.) was developed and supplied by Dr. Grosser (Analytical Research Laboratory, Bayer AG). The column packing material consisted of silica gel modified with an optically active form of poly(triphenylmethylmethacrylate) and the mobile phase was methanol (hplc grade, 0.5 ml/min). Individual extracts were dissolved in hexane (hplc grade) and aliquots containing 10 ug injected. Reference mixtures of different proportions of 1R and 1S triadimefon gave retention volumes of 4.05 ml and 6.0 ml respectively with linear u.v. response.

Triadimenol enantiomer content

Extracts, dissolved in methanol (hplc grade, 1 ml), were analysed using a published glc method (Clark & Deas 1985). As corroborative evidence for the glc enantiomeric composition data, samples were analysed for diastereoisomer content using a published tlc method (Deas & Clifford 1982) in conjunction with an Isomess RITA 4 radio-tlc linear analyser.

RESULTS

Stability of triadimefon enantiomers

The total recoveries of [^{14}C]-labelled material were of the same order as obtained in similar experiments (Buchenauer & Grossman 1982; Deas et al. 1986). For samples taken after incubation periods up to, and including 16h, returns of 85 - 112% were obtained. After longer periods of incubation, levels of recovery dropped to 75 - 85%. No difference was observed between the recoveries of the two enantiomers.

Analyses of extracts from buffered solutions of individual enantiomers showed that at pH4 and 5 no detectable conversion of 1R to 1S triadimefon, or vice versa, had occurred within 72h. At higher pH values racemisation of both the 1R and 1S enantiomers did occur, producing mixtures of the two enantiomers. Rates of racemisation increased with increasing pH (see Table 1) and, at pH 8, an equilibrium was reached within 16h, and possibly even within 7h. Statistical analysis of the data showed that they were best described by equations associated with first order reactions, viz: $dc/dt = -kc$ where c is the rate constant for

that reaction. No significant difference between the rates of racemisation of the two enantiomers at any given pH was observed and it was possible, therefore, to combine the data for the 1R and 1S curves, thereby deriving more precise values for the rate constants (see Table 2).

TABLE 1

Enantiomeric composition (%) of triadimefon extracted from buffered aqueous solutions of individual enantiomers incubated at 25°C

pH	Enantiomer	Incubation period (h)								
		0	0.5	2.0	4.0	7.0	16.0	24.0	48.0	72.0
4.0 and 5.0	R	100				100	100	100	100	100
	S	0				0	0	0	0	0
	R	0				0	0	0	0	0
	S	100				100	100	100	100	100
6.0	R	100			100	100	75	79	51	
	S	0			0	0	25	21	49	
	R	0			4	19	9	11	25	
	S	100			96	81	91	89	75	
7.0	R	100	100	84	79	74	58			
	S	0	0	16	21	26	42			
	R	0	0	14	15	43	35			
	S	100	100	86	85	57	65			
8.0	R	100	89	70	58	49	52			
	S	0	11	30	42	51	48			
	R	0	21	25	40	45	49			
	S	100	79	75	60	55	51			

Stability of triadimenol enantiomers

Recoveries (89 - 101%) of added [^{14}C]-triadimenol in extracts from buffered solutions were comparable with those obtained previously (Deas et al. 1984a,b). Analyses of extracts by tlc (for diastereoisomer content) and by glc (for enantiomer content) were in agreement and showed that after 72h incubation there had been no detectable change in the stereochemical composition of the added compound at any of the three pH values. Analyses of extracts from fungal cultures also failed to detect any change in enantiomeric composition or any oxidation to triadimefon.

The initial pH of the cultures was 5.6. After incubation for three days, when the enantiomers were added, pH values ranged from 3.5 (B. cinerea) to 5.5 (P. teres). After a further two days incubation the pH of the B. cinerea cultures remained at 3.5 but for all others it was 5.5 - 5.7. The presence of triadimenol enantiomers had no effect either on pH values or dry weight of the mycelia.

TABLE 2

Rate constants for the racemisation of triadimefon in aqueous solutions of different pHs

Enantiomer	pH	k[1]	s.e.[2]	$t_{0.5}$[3]
1R	8.0	0.485		1.43
1S	8.0	0.415		1.67
1R/S[4]	8.0	0.452	0.042	1.53
1R	7.0	0.124		5.59
1S	7.0	0.142		4.88
1R/S	7.0	0.132	0.015	5.24
1R	6.0	0.032		21.48
1S	6.0	0.015		46.70
1R/S	6.0	0.023	0.004	29.82

where 1 = rate constant (/h)
2 = standard error; 19 d.f.
3 = half life of the reaction (h)
4 = combined 1R and 1S curves

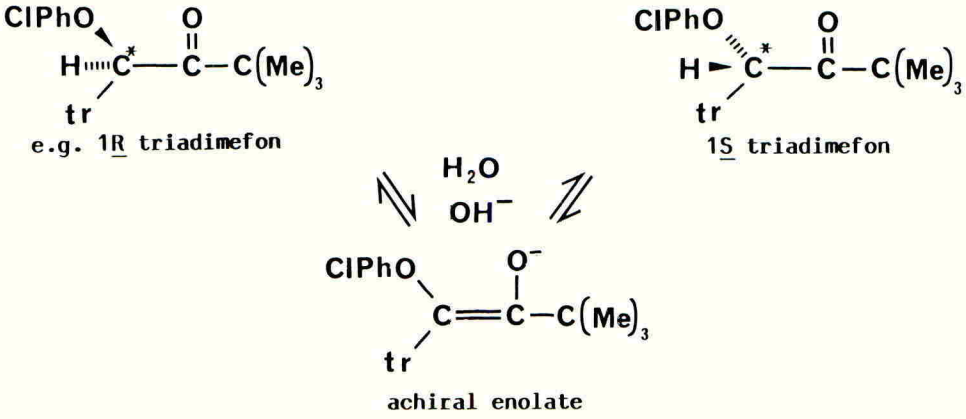
DISCUSSION

The present data confirm that racemisation of triadimefon enantiomers occurs readily in aqueous solutions at pH 6 and above. The 1R and 1S enantiomers racemise at equal rates and solutions of either will change in composition until a 1 : 1 equilibrium mixture of both is attained. Triadimefon is marketed as a 1 : 1 mixture of the two enantiomers and, therefore, solutions of the commercial product will show no apparent change in composition at any pH, although a dynamic equilibrium will have been established at pH values of 6 or greater. This stereochemical instability of triadimefon enantiomers in aqueous environments is the most likely reason for the similar level of activity shown by them in laboratory assays of fungitoxicity. Triadimefon enantiomers are liable to undergo racemisation in fungal metabolism studies either in the culture medium or inside the fungal mycelium, the internal pH of which may differ from that of the medium. Furthermore, triadimefon is also likely to undergo metabolic reduction to give mixtures of triadimenol enantiomers (Deas *et al*. 1984a,b; 1986) and possibly also oxidation to the primary alcohol (Deas & Clifford 1982). Analytical data on the products present after incubating triadimefon enantiomers with fungi could, therefore, be difficult to interpret and for this reason such studies were not undertaken.

In contrast to triadimefon, solutions of triadimenol enantiomers were stable throughout the pH range 4 - 8.

Racemisation of triadimefon probably proceeds via a base-catalysed enolisation of the keto- group, giving an achiral enol which subsequently reverts to both enantiomers of the keto- form (see Fig. 2). The stability of the triadimenol molecule under similar conditions is explained by its inability to undergo this reaction. The widely

different levels of fungitoxicity shown by the four enantiomers of triadimenol indicates that they also maintain stereochemical stability under the conditions of our laboratory assays.



* = chiral centre
tr = 1,2,4-triazol-1-yl

Fig. 2. Postuled mechanism of triadimefon racemisation.

In a previous investigation (Deas & Clifford 1984), using static cultures of Coriolus versicolor and Rhizoctonia solani, oxidation of triadimenol to triadimefon was observed. No evidence for this was found in the present study. The presence of an oxidation-reduction system in a metabolising fungus could provide a route for an apparent epimerisation of triadimenol enantiomers e.g. 1R,2S triadimenol ---(oxidation)---> 1R triadimefon ---(reduction)---> 1R,2R triadimenol. If the 1R triadimefon also underwent racemisation to the 1S enantiomer then both the 1S,2R and 1S,2S triadimenols could be formed.

It appears, from work to be reported at this Conference by Drs. T. Clark and K. Vogeler, that the chiral and metabolic stability of triadimenol enantiomers in higher plants is somewhat lower than in aqueous solutions or fungal cultures.

ACKNOWLEDGEMENTS

The authors would like to thank Long Ashton colleagues Drs. N.H. Anderson and P. Brain for useful discussions and Mrs. S.J. Kendall for technical assistance. We also thank Bayer AG for helpful advice and the provision of materials. Long Ashton Research Station is a grant-aided Institute funded by the Agricultural and Food Research Council.

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PIRIMIPHOS-METHYL - METABOLISM IN RUMINANT AND POULTRY

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ABSTRACT

The nature of the radioactive residues found in tissues, milk and eggs following the feeding of ^{14}C-radiolabelled pirimiphos-methyl to a goat and hens has been investigated.

In both species the compound was readily metabolised, mainly by hydrolysis with loss of the phosphorothioate ester side chain and N-dealkylation; the products formed were subsequently conjugated with endogenous compounds. Separation of these polar compounds from aqueous solutions and dissolved high molecular weight biological material, using non-ionic polymeric resin (Amberlite XAD4) and gel permeation chromatography, has allowed quantitative analysis of the radioactive residues using thin layer chromatography and automatic linear scanning.

INTRODUCTION

Pirimiphos-methyl [O-(2-diethylamino-6-methylpyrimidin-4-yl)O,O-dimethyl phosphorothioate] 'Actellic' is an outstanding broad spectrum insecticide used to control a wide range of pests in a variety of agricultural, horticultural and forestry conditions.

The properties of pirimiphos-methyl,

- low mammalian toxicity
- long term activity on surfaces
- activity against most organo-phosphorus resistant insect strains
- useful fumigant effect
- effectiveness over a wide temperature range

make it particularly interesting as a stored product insecticide for both short and long term control of insects and mites in stored grain. When the insecticide is used in this way, residues of pirimiphos-methyl will be ingested by animals when the treated grain is used for animal feed.

In this paper we describe the methodology and report the results from an investigation of the nature of the residues in edible tissues, milk and eggs following the feeding of ^{14}C-labelled pirimiphos-methyl to a goat and hens.

MATERIALS AND METHODS

Pirimiphos-methyl radiolabelled with ^{14}C in the 2 position of the pyrimidine ring (Figure 1) was used for the feeding studies.

*denotes the position of the ^{14}C-radiolabel

Fig. 1. Structure of Pirimiphos-methyl

One goat and three hens were dosed with ^{14}C-pirimiphos-methyl at a rate equivalent to c.50 ppm in the diet for 7 and 14 consecutive days respectively. The compound was administered absorbed onto standard diet in sealed gelatin capsules. Levels of chemical used were calculated on the basis that a 2 kg hen consumes approximately 100 g food/day while a goat will consume on average 4% of its body weight in dry matter over the same period. Both species were kept in metabolism cages throughout the study and allowed food and water ad-libitum. The health of the animals was also carefully monitored. Excreta and eggs were collected daily from the hens. The goat was milked twice in a 24 hour period and the faeces and urine collected at the end of each day. Sixteen hours after administration of the final dose the animals were sacrificed and a range of tissues taken for analysis.

The radioactive content of liquid samples was determined using liquid scintillation counting (LSC). Solid samples were combusted (Packard model 306 sample oxidiser) and assayed using LSC.

A generalised scheme of analysis, used to investigate the nature of the radioactive residues in the tissues, is shown in Fig. 2. Significant levels of radioactivity (c.40% of the total radioactive residue) remained unextracted in the livers from both species following the initial extraction. Figure 3 shows the generalised scheme of analysis used for the investigation of the unextracted radioactive residue.

Characterisation of the residues in the fractions was carried out using thin layer chromatography in a range of solvent systems. Quantification of the amount of radioactivity co-chromatographing with authentic reference markers was achieved using an automatic TLC linear analyser (Isomess IM 3000).

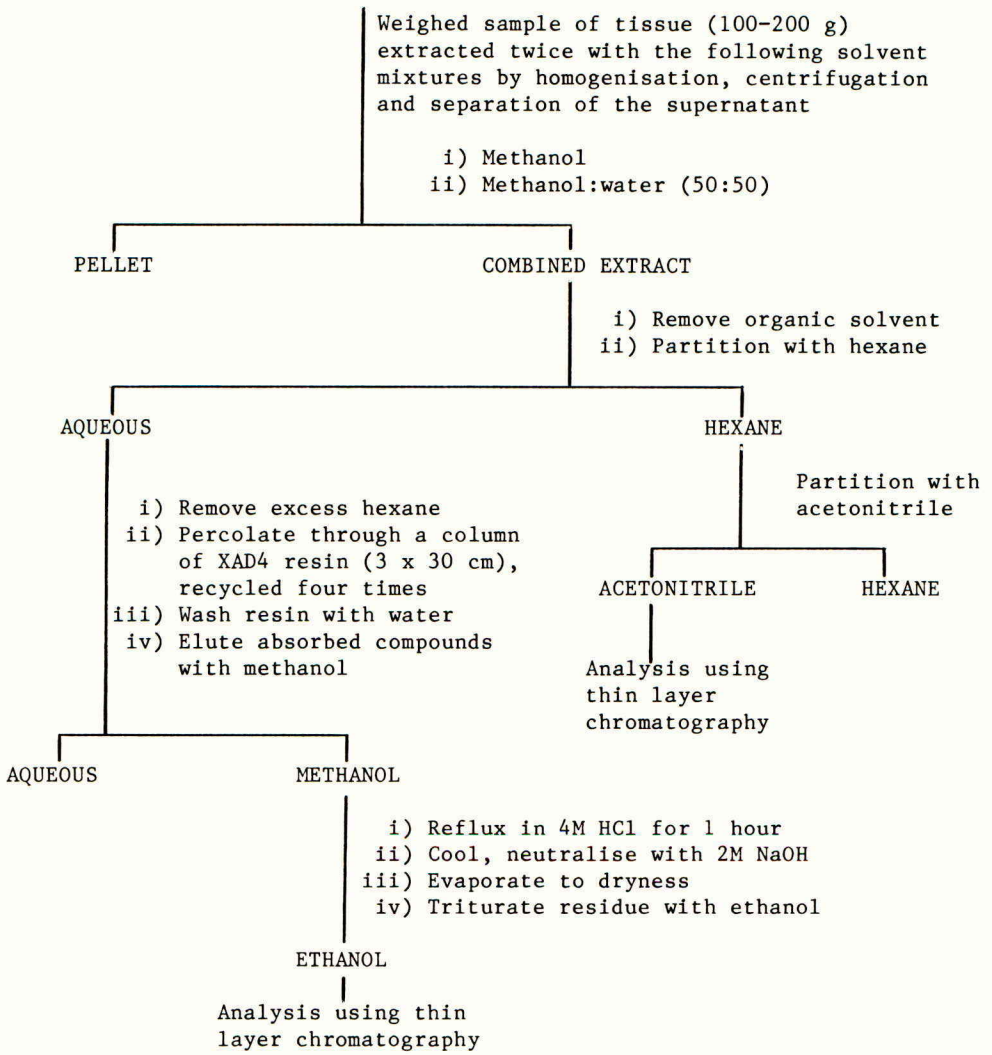
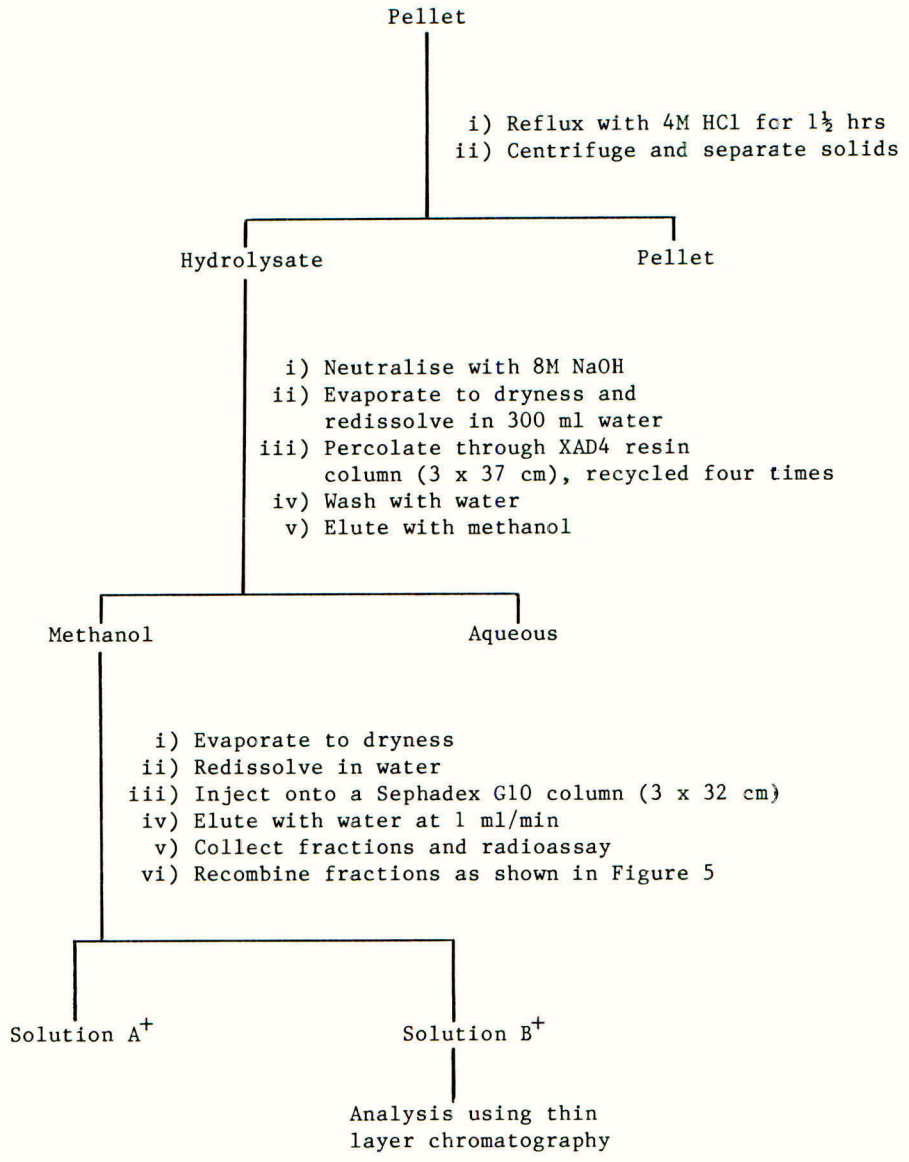


Fig. 2. Generalised extraction and fractionation scheme used to characterise the radioactive residues in tissues (except fat)



+See Fig. 5.

Fig. 3. Extraction and Fractionation Scheme Used to Characterise the Radioactivity not extracted from the Liver

RESULTS AND DISCUSSION

At sacrifice 89 and 97% of the administered radioactivity had been excreted by the goat and hens respectively. Radioactive residues in tissues, milk and eggs are shown in Tables 1 and 2.

TABLE 1

Radioactive Residues in the Tissues and Milk of the Goat (Expressed as mg/kg Pirimiphos-Methyl Equivalents) Equivalents)

Tissue	Radioactive Residue mg/kg
Meat	
Hindquarter	0.044
Forequarter	0.044
Fat	
Subcutaneous	0.060
Peritoneal	0.074
Liver	0.31
Kidney	0.50
Milk	0.16

TABLE 2

Radioactive residues in tissues and eggs of hens (Expressed as mg/kg Pirimiphos-Methyl Equivalents)

Tissue	Radioactive Residue mg/kg
Liver	0.20
Peritoneal fat	0.09
Subcutaneous fat	0.11
Leg muscle	0.67
Breast muscle	1.30
Egg yolk	0.19
Egg albumen	0.16

Characterisation of the Radiactive Residues in Tissues, Eggs and Milk

Pirimiphos-methyl is readily metabolised by hydrolysis resulting in the loss of the phosphorothioate side chain and N-dealkylation giving compounds III, IV and V (Fig. 4). These polar metabolites do not partition reproducibly into organic solvents from an aqueous solution containing biological co-extractives, despite pH control. Amberlite XAD4 resin quantitatively removes compounds III, IV and V and their conjugated forms from aqueous solution. Absorbed compounds were recovered from the resin with methanol.

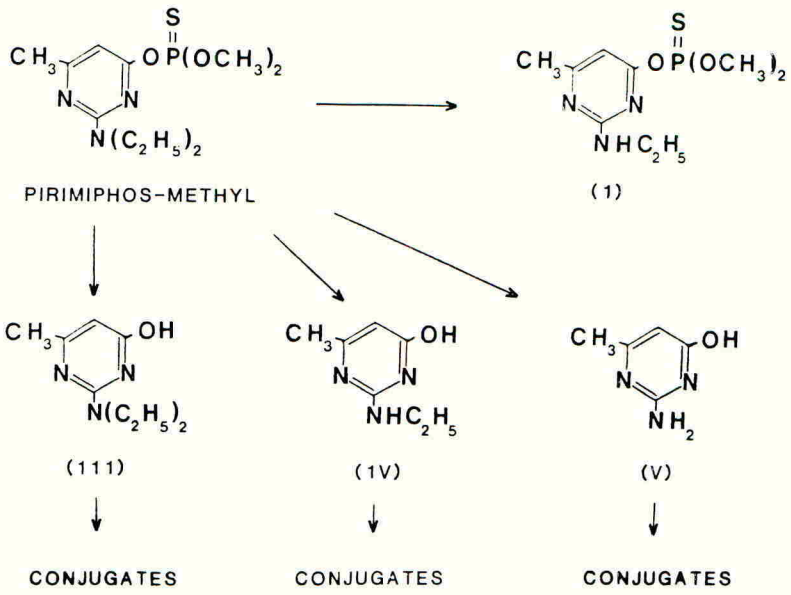


Fig. 4. Proposed biotransformation pathway for pirimiphos-methyl

Hydrolysis of the phase II metabolites was investigated using 2 M NaOH, 4 M HCl and β glucuronidase (Helix Pomatia; Sigma Chemicals Ltd). The products of hydrolysis were qualitatively the same in each hydrolysate; 4 M HCl being the most effective.

Hydrolysis of unextracted liver residue with 4 M HCl also solubilised high levels of endogenous material. The high molecular weight fraction of this was removed using gel permeation chromatography. The reconstructed chromatogram in Fig. 5 shows the distribution of radioactivity eluted from the column. Solution B was analysed by thin layer chromatography and shown to contain compounds III, IV and V. A smaller amount of radioactivity was associated with the high molecular weight fraction Solution A.

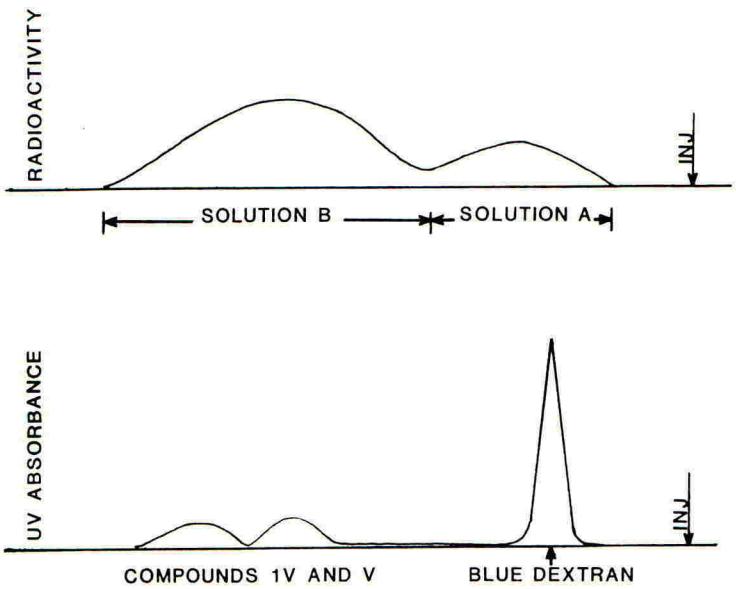


Fig. 5. Reconstructed gel permeation chromatogram of the liver hydrolysate

Results of the analysis of the goat tissues and milk shows that pirimiphos-methyl and O-(2-ethylamino-6-methylpyrimidin-4-yl)-O,O-dimethyl phosphorothioate (compound I) were the major components in the fat (55.2% and 17.1% respectively). The major radioactive components found in the remaining tissues and the milk were 2-ethylamino-6-methylpyrimidin-4-ol (compound IV) and 2-amino-6-methylpyrimidin-4-ol (compound V). The percentages of the total radioactive residue in the tissues and milk associated with compound IV were: meat (23.5%), liver (21.3%), kidney (36.9%) and milk (31.8%). Figures for the liver and kidney express a summation of both free and conjugated forms of compound IV. Similarly for compound V: meat (20.1%), liver (9.1%), kidney (9.0%) and milk (14.3%). 2-diethylamino-6-methylpyrimidin-4-ol (compound III) was present at generally lower levels: meat (4.7%), liver (10%), kidney (14.0%), milk (4.5%). In the kidney compound III existed in a predominantly conjugated form.

In the tissues and eggs of the hens, pirimiphos-methyl was found to be the major radioactive component in the fat (72.5%) and was also present in the egg yolk (9.5%). Compounds IV and V were the major components in the egg yolk (33.8% and 11.3% respectively), albumen (43.2% and 21.6% respectively) and liver (24.0% and 20.1% respectively). Conjugated forms of compounds IV and V were present in the liver while conjugated compound V was the major component in the muscle (c.71%). Although the total radioactive residue in the breast muscle is approximately twice that in the leg muscle a similar profile of the radioactive components was observed.

CONCLUSIONS

In a typical ruminant and poultry species pirimiphos-methyl is readily hydrolysed by loss of the phosphorothioate side chain and N-dealkylation. The proposed biotransformation pathway is shown in Fig. 4.

The use of non-ionic polymeric resins (XAD4) and gel permeation chromatography has facilitated characterisation of these polar, highly water soluble, phase 1 and phase 11 metabolites by thin layer chromatography.

ACKNOWLEDGEMENTS

The authors acknowledge Huntingdon Research Centre, Huntingdon, Cambs. for the work they carried out to investigate the metabolism of pirimiphos-methyl in hens.

CLOFENTEZINE: DEGRADATION AND MOBILITY IN SOILS/SEDIMENTS.

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ABSTRACT

[^{14}C]-Radiolabelled clofentezine was applied at approximately field rate to three soil types, clay, loamy sand and clay loam. The treated soils were incubated under aerobic and anaerobic conditions for up to 1 year at 25°C and a moisture content of 50% M.H.C.

Rapid degradation of clofentezine in the three soils resulted in 50% loss of pesticide within 4-8 weeks. The principle degradative route, under aerobic conditions, was hydrolytic cleavage of the tetrazine ring leading to the formation of 2-chlorobenzoic(2-chlorobenzylidene) hydrazide. Further breakdown occurred with up to 50% of the applied radioactivity being mineralised to $^{14}CO_2$.

More rapid degradation of the parent compound occurred when the radiolabelled pesticide was applied to surface water of sediment/water systems. In two sediment types 'half-lives' of 2-7 days were observed with substantial degradation to $^{14}CO_2$.

In a range of mobility studies, using both active ingredient and formulated products, clofentezine was found to be of extremely low mobility in all soil types. This is in general agreement with predicted mobility based on the octanol/water partition coefficient.

INTRODUCTION

Clofentezine (Figure 1) is an acaricide having both ovicidal and larvicidal activity. Its principle use is in orchard situations on apples and pears but it is also being used on citrus, vines and cotton. As part of the evaluation of the product's safety in the environment the degradation of [^{14}C] labelled clofentezine has been studied in a range of soil types incubated under laboratory conditions, and in river water with associated sediments. In addition, the mobility of the compound has been examined using soil columns and soil thin layer chromatography.

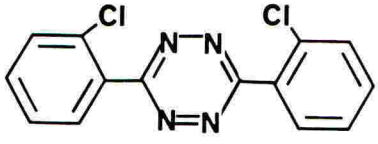

Figure 1 (Clofentezine (3,6-bis(2-chlorophenyl)-1,2,4,5-tetrazine)

MATERIALS AND METHODS

Degradation Studies

Soils

[^{14}C]-tetrazine-ring-labelled clofentezine was applied at approximately field rate to three soil types, clay loamy sand and clay loam. Degradation was investigated under aerobic, anaerobic and sterile conditions. The soils were incubated at 25°C in the dark and continuously flushed with either carbon dioxide-free moist air at a rate of up to 10 ml/min for aerobic treatments (sterilised by in-line filters for sterile soils) or nitrogen gas for the flooded (anaerobic) soil treatments.

Sediment water

Surface water/sediment microcosms were prepared using two river sediments (a sandy clay loam and a clay loam) and their corresponding waters. [^{14}C]-Clofentezine was applied to the surface water at a rate equivalent to 1.0 kg ai/ha and the microcosms incubated at 20°C in the dark, with aeration using carbon dioxide free moist air.

Volatile products evolved from both the soils and sediment/water microcosms were 'trapped' in appropriate solvents.

Mobility Studies

The mobility of [^{14}C]-clofentezine has been investigated in comparison with three reference standards (atrazine, 2,4-D and paraquat) on soil thin layer plates using four contrasting soil types, sandy loam, silt loam, clay and a sand with less than 1% organic matter. The plates were eluted with 0.01M $CaCl_2$ by ascending chromatography with radiodetection by autoradiography and radiochromatographic scanning. The mobility has also been studied using 30 cm long x 5 cm i.d. columns with the same contrasting soil types. The columns were leached with 0.01 M $CaCl_2$ for 30 days with 34 ml/day. The leachate was collected daily for analysis.

Analysis

Quantitative

A balance of radioactivity was determined for each sample. Radioactivity in soils and sediment/water was removed by soxhlet extraction using polar and non-polar solvents. The distribution of radioactivity in sediment and water was separately determined. Unextracted ('bound') radioactivity was determined by oxidation of subsamples of soil and sediment and trapping the [^{14}C]-carbon dioxide released for quantification by liquid scintillation counting.

Qualitative

Following concentration of extracts, the distribution of clofentezine and its degradation products was determined by thin layer chromatography (tlc) in two solvent systems using authentic unlabelled reference compounds as markers. The relative positions

of the reference compounds on tlc were located by visualisation under UV light and the radiolabelled products by exposure to no-screen x-ray film. The major degradates were further characterised by high performance liquid chromatography (hplc).

RESULTS

Fate in soil and sediment/water

Clofentezine was degraded in all three soils under aerobic conditions with a 'half-life' of approximately 4,6 and 8 weeks in the clay, loamy sand and clay loam respectively. In the surface water/sediment microcosms degradation was much more rapid with 'half-lives' of less than 1 week (Fig 2).

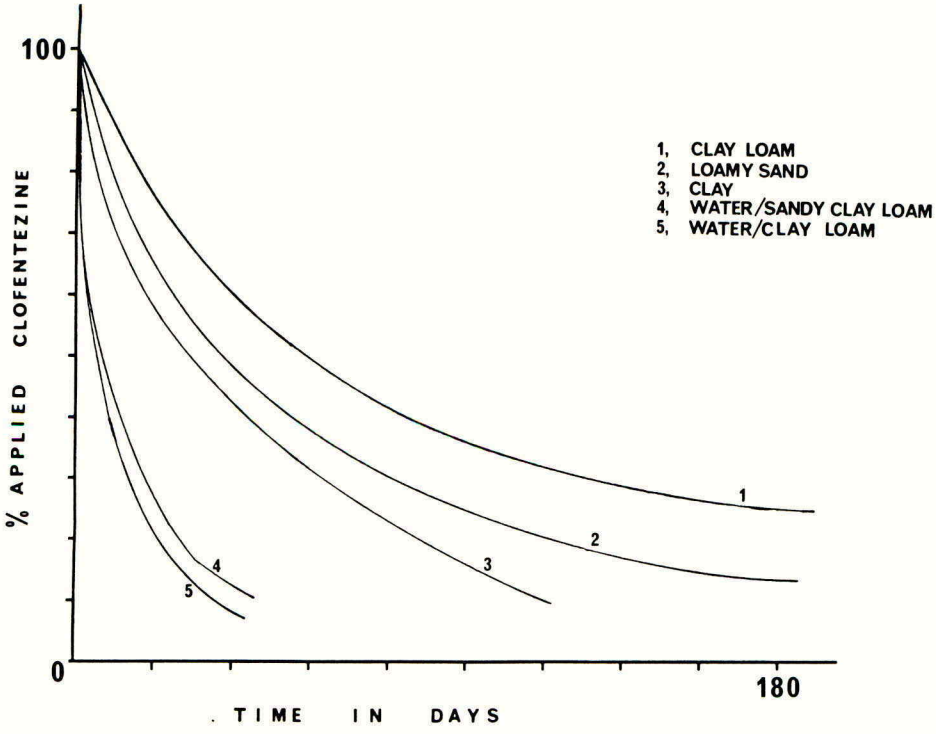

FIG. 2 The rate of degradation of clofentezine in soils and sediment/water microcosms

Degradation in both systems proceeded via hydrolytic cleavage of the tetrazine ring leading to the formation of a major metabolite, 2-chlorobenzoic(2-chlorobenzylidene) hydrazide. Further degradation led to the formation of several other minor metabolites with ultimate mineralisation to radiolabelled carbon dioxide. Substantial amounts of which (up to 50% of the applied radioactivity from aerobic soils) were evolved from both aerobic soils and the sediment/water microcosms (Fig. 3).

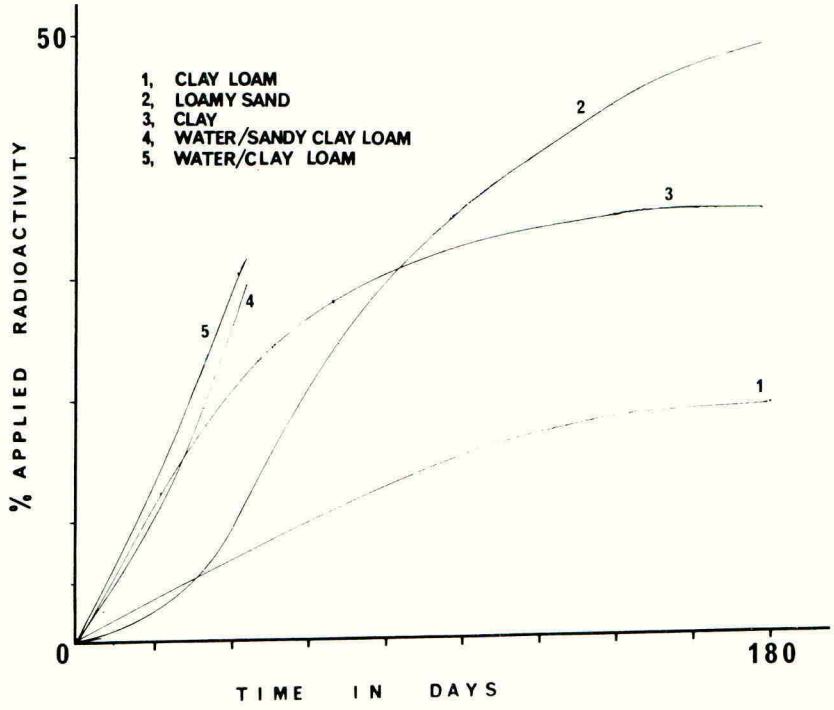

FIG. 3 Evolution of $^{14}CO_2$ from clofentezine treated soils and sediment water microcosms

The rate of mineralisation was significantly reduced under anaerobic soil conditions and virtually absent in sterile soils. Unextracted ('bound') residues increased with time reaching a plateau between 1 and 3 months depending on soil type.

Mobility

No movement of [^{14}C]-clofentezine was detected either on soil TLC plates or in soil columns. On the Helling scale (Helling 1971) clofentezine was immobile (class 1) in comparison with atrazine and 2,4-D which were of intermediate mobility and mobile (classes 3 and 4) respectively. No radioactivity was detected in the leachate from the soil column study.

DISCUSSION

The degradation of clofentezine in soil is mediated by the activity of micro-organisms as shown by the virtual absence of decay under sterile conditions, hence it is unlikely to persist under agricultural conditions. Because of its rapid hydrolysis in natural waters it is also unlikely to accumulate in sediments or aquatic organisms.

Data from the mobility studies are in general agreement with the low mobility of clofentezine predicted from the octanol/water partition coefficient where log P = 3.1. Log P has been related to log Q (soil organic matter) water partition coefficient by Briggs (Briggs 1973) where log Q = (0.524 log P) + 0.618. For clofentezine log Q = 2.242 and Q = 175. This equation is however, only valid for unionised chemicals. As clofentezine was in fact immobile, rather than of low mobility, in leaching systems it is likely that ionic binding to clay and organic matter is a contributing factor to its immobility.

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BIOLOGICAL PROFILE OF SAN 619 F AND RELATED EBI-FUNGICIDES

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ABSTRACT

Ergosterol biosynthesis inhibitors (EBIs) are broad spectrum fungicides against diseases caused by fungi of almost any class of pathogens except Peronosporales. In respect to their spectrum of activity, we recognized four types of EBIs: (1)fenpropimorph-, (2)prochloraz-, (3)propiconazole-, (4)SAN 619 F-type. SAN 619 F, fenpropimorph and bitertanol penetrated into the plant tissue very rapidly. Translocation of EBIs in the plant was in the transpiration stream and therefore acropetally; there was no export from treated into untreated leaves. Strong systemicity was observed with SAN 619 F, triadimenol, propiconazole, penconazole and fenpropimorph. Against powdery mildew and rust, preventive activity lasted about 12 days with SAN 619 F, and 4 to 8 days with flusilazole, triadimenol, fenarimol and propiconazole. Strong curative and interesting eradicative activities were found with fenpropimorph, triadimenol, SAN 619 F and propiconazole. Triadimefon was less effective against field strains of powdery mildew; propiconazole, penconazole and fenarimol were intermediate; whereas triadimenol, SAN 619 F and fenpropimorph were almost equally active against all strains. Triazoles but not morpholines were less active under cool and humid conditions.

INTRODUCTION

EBI-fungicides are subdivided into two groups according to their biochemical mode of action: (1) Sterol C-14 demethylation inhibitors (DMIs) affecting cytochrome P-450 enzymes (pyrimidines like fenarimol, nuarimol; imidazoles like prochloraz; triazoles like bitertanol, triadimefon, triadimenol, propiconazole, penconazole, flutriafol, flusilazole, SAN 619 F; piperazines like triforine); (2) Inhibitors of sterol delta 14(15)-reduction and delta 8/7 isomerisation (morpholines like fenpropimorph, tridemorph). Irrespective of the similarities of their biochemical modes of action, EBI fungicides vary significantly in their biological behavior against pathogens and their hosts. The differences between EBI-molecules in (1) the spectrum and level of activity, (2) the penetration and systemicity in the plant, (3) the duration of preventive, curative and eradicative activity and (4) the response to different fungus strains at different climatic conditions are shown in this paper.

SPECTRUM AND LEVEL OF ACTIVITY (PREVENTIVE ACTIVITY, Tables 1, 2, 3)

SAN 619 F is very active against different pathogenic fungi on wheat, barley and rice (Table 1). Excellent activity was found against all rust and powdery mildew diseases (Puccinia and Erysiphe), rust control being much better with SAN 619 F and powdery mildew control being about equal to that of all the other molecules. Against Septoria and Helminthosporium, there is important supplementary control with SAN 619 F and most of the other molecules (except fenpropimorph), whereas Rhizoctonia and Pellicularia are controlled effectively only by SAN 619 F. Rather weak but still interesting levels of control were found with SAN 619 F against Pseudocercosporella (wheat), Pyrenophora (barley) and Pyricularia (rice), but other molecules

like prochloraz were more active. Triadimefon was always inferior to triadimenol (data not shown).

Like the other EBI-molecules (Table 2), SAN 619 F is equally active against powdery mildew on grape and apple (Uncinula, Podosphaera); downy mildew on grape (Plasmopara) was not controlled (data not shown). SAN 619 F is very active against apple scab (Venturia; tested on leaves); it is somewhat better than bitertanol, penconazole and fenarimol. SAN 619 F was the best compound on Monilinia (tested on apple fruits) and Hemileia (coffee rust). Additional in vitro tests indicated very promising results with SAN 619 F against Taphrina, Stereum and Fomes (Gisi et al. 1986).

In field crops and ornamentals, a variety of important pathogenic fungi were controlled extremly well with SAN 619 F which was generally better than the other molecules (Table 3). This includes especially rust diseases (Uromyces, Puccinia) and Cercospora on sugar beet and peanuts. Closest to SAN 619 F are flusilazole and penconazole. Against cucumber powdery mildew (Sphaerotheca), all molecules including SAN 619 F were extremely active. Neither Pythium on cucumber (damping off) nor Phytophthora on potato were controlled by any of the molecules. Interesting levels of activity can be expected from SAN 619 F, flusilazole and propiconazole against Alternaria, whereas the Botrytis activity of SAN 619 F (data not shown) is too weak for practical conditions. Additional in vitro tests with SAN 619 F (Gisi et al. 1986) revealed extremely interesting results against Sclerotium, Rhizoctonia and promising activity against Sclerotinia, Phoma, Aphanomyces and Thielaviopsis, whereas there was not much activity against Fusarium species.

Thus, EBI-molecules control diseases caused by fungi of almost any class of pathogens except Peronosporales and to a certain degree also Botrytis. Nevertheless, there are considerable differences in the spectrum of activity among the tested molecules. Evaluating the molecules from a biological point-of-view regardless of their chemical structure, four different types may be distinguished each with typical strong points: (1) **fenpropimorph-type** (good activity against powdery mildews and rusts); (2) **prochloraz-type** (excellent activity against Septoria, Helminthosporium and Pseudocercosporella, good activity against Pyricularia, additional activity against powdery mildews and rusts); (3) **propiconazole-type,** including also penconazole and flutriafol (excellent activity against powdery mildews, good activity against Monilinia and rusts, additional activity against Septoria, Helminthosporium, Cercospora, Venturia, Pyricularia; (4) **SAN 619 F-type** (excellent activity against powdery mildews, Monilinia, Cercospora, Venturia, rusts, good activity against Rhizoctonia, Pellicularia, Sclerotium, additional activity against Septoria, Helminthosporium, Pyricularia). The other molecules are located somewhere in between the four types: triadimenol/triadimefon between type 1 and 4; bitertanol, fenarimol between type 2 and 3 and flusilazole between type 2 and 4.

CROP TOLERANCE

When tested at dosages higher than 80 mg/l under greenhouse conditions, SAN 619 F showed the same symptoms of phytotoxicity on wheat, grape, apple, cucumber, bean and tomato as other triazole fungicides (stunting, crinkling, foliage reduction, sometimes also burning). The safest molecules were bitertanol, prochloraz and fenpropimorph. When used at an appropriate dosage for disease control, SAN 619 F is not expected to cause any significant problems of phytotoxicity.

Table 1: Preventive fungicide activity of different EBI-molecules against diseases on cereals under greenhouse conditions, expressed as EC 90 (mg/l)

Fungus/plant	SAN 619 F	flusil-azole	propi-con-azole	flu-tria-fol	triadi-menol	pro-chlo-raz	fen-propi-morph
wheat							
Pseudocerc. herpotrich.	35	19	40	32	33	8	91
Rhizoctonia solani	60	>900	>900	>900	128	>900	630
Septoria nodorum	10	9	8	11	9	5	240
Erysiphe graminis	2	2	3	2	3	5	14
Puccinia striiformis	2	7	7	8	7	8	10
Puccinia triticina	2	6	9	9	8	22	29
Puccinia graminis	2	9	8	11	8	11	18
barley							
Erysiphe graminis	7	4	9	10	5	8	9
Helminthosporium sativum	9	9	25	12	35	15	350
Pyrenophora graminea	130	29	23	>900	720	19	150
rice							
Pellicularia sasakii	12	390	70	260	>900	>900	>900
Pyricularia oryzae	16	13	17	33	>900	1	28

Table 2: Preventive fungicide activity of different EBI-molecules against diseases on grapes (gr), apples (ap) and coffee (co) under greenhouse conditions, expressed as EC 90 (mg/l)

Fungus/plant	SAN 619 F	flusil-azole	propi-con-azole	pencon-azole	tria-di-menol	biter-tanol	fena-rimol
Uncinula necator (gr)	2.0	2.2	3.3	2.0	3.0	2.3	2.0
Podosphaera leucotr. (ap)	2.1	2.2	3.3	2.0	2.4	2.2	3.0
Venturia inaequalis (ap)	0.5	0.3	3.0	2.7	12	3.5	2.2
Monilinia fructicola (ap)	8	16	13	13	27	51	72
Hemileia vastatrix (co)	2	7	8	5	6	8	12

Table 3: Preventive fungicide activity of different EBI-molecules against diseases on field crops and ornamentals under greenhouse conditions, expressed as EC 90 (mg/l)

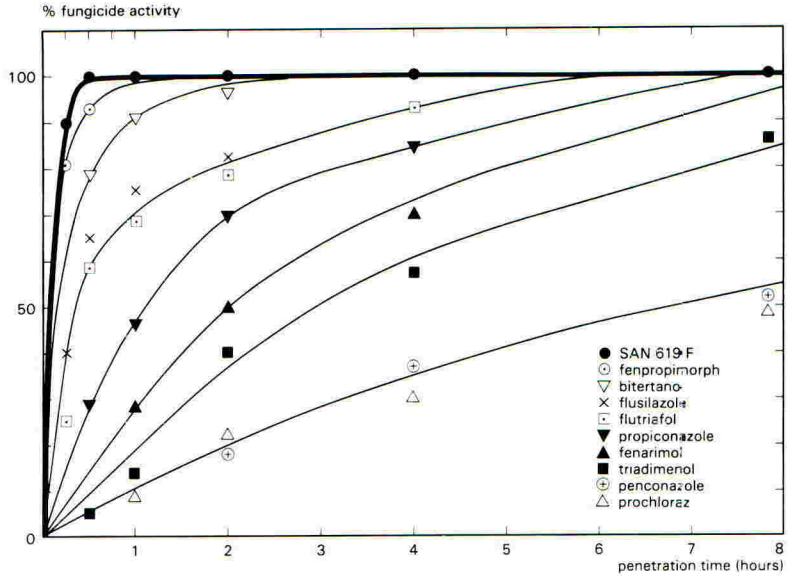
Fungus/plant	SAN 619 F	flusil-azole	propi-con-azole	pencon-azole	triadi-menol	biter-tanol	fena-rimol	pro-chlo-raz
Sphaerotheca/cucumber	0.6	1.4	0.9	1.5	2.4	0.8	2.4	2.4
Uromyces/bean	0.3	0.7	3.2	2.5	2	1.5	8	13
Alternaria/chin. cabbage	29	36	70	>900	128	96	230	124
Cercospora/sugar beet	3	7	30	9	45	24	77	37
Cercospora/peanuts	2	14	8	31	24	6	6	98
Puccinia/pelargonium	4	7	11	8	9	440	147	360
Puccinia/snap dragon	2	6	9	6	7	24	9	22

Note: EC 90 values are concentrations (mg a.i./l) providing 90% disease control. EC 90 values should be compared line by line rather than column by column. Values within a line are significantly different if they vary by more than a factor of 2.

PENETRATION INTO THE LEAF (Figure 1)

The speed of penetration of SAN 619 F and other EBI-molecules (2 mg/l) into bean leaves was tested in a bioassay. SAN 619 F and fenpropimorph penetrate rapidly into the leaf providing full disease control 30 minutes after application, followed by bitertanol (90 minutes), then the other compounds.

Figure 1 Penetration time of 2 mg/l of EBI-molecules into bean leaves infected with *Uromyces appendiculatus*



SYSTEMICITY (Tables 4 and 5)

When SAN 619 F was applied to the soil, it was readily taken up by the roots and was translocated to the leaves (root/leaf, Table 4), where it controlled powdery mildew at very low concentrations. Triadimenol and fenarimol were also very systemic in this test. When individual droplets of formulated fungicide were applied across the upper leaf surface (Table 4), the acropetal (acro) and basipetal (basi) distribution in the leaves could be studied. SAN 619 F was almost equally well distributed in both directions. Also triadimenol, fenpropimorph, propiconazole and penconazole (the last two only in the cucumber/Sphaerotheca system) gave good results indicating clear systemic behavior. Flutriafol, fenarimol and flusilazole showed medium, prochloraz and bitertanol little systemic behavior. Similar results were obtained, when a drop of fungicide was applied to the apical bud with subsequent acropetal movement of the molecules into the corresponding leaf (bud/leaf, Table 4). The strongest systemicity was expressed with SAN 619 F and triadimenol, followed by propiconazole, penconazole, flutriafol, fenarimol, flusilazole and fenpropimorph; no systemicity was found with bitertanol and prochloraz. Systemicity may be related to the crop tolerance because the least systemic EBI-molecules also showed the least phytotoxic symptoms.

The fungicide translocation within an entire plant is very important to field performance. Cucumber plants were treated with the fungicides either as stem application between the fourth and the fifth leaf or as a drop ap-

plication on the fourth and fifth leaf (Table 5). With stem application, there was very clearly only acropetal and no basipetal translocation of the fungicides, with SAN 619 F being more systemic than both triadimefon and propiconazole, whereas with the leaf application, there was no export from treated into untreated leaves of any of the tested fungicides.

Table 4: Fungicide activity of different EBI-molecules in untreated plant parts resulting from systemic behavior in cucumber and bean plants infected with Sphaerotheca fuliginea and Uromyces appendiculatus, respectively, under greenhouse conditions, expressed as EC 90 (mg/l)

	cucumber/Sphaerotheca				bean/Uromyces		
molecule	root /leaf	leaf acro	leaf basi	bud /leaf	leaf acro	leaf basi	bud /leaf
SAN 619 F	0.4	2	4	10	3	9	16
flusilazole	nt	49	>900	28	190	>900	670
propiconazole	4.6	5	13	17	64	>900	730
flutriafol	nt	11	>900	27	74	350	53
penconazole	2.5	7	36	20	360	610	>900
bitertanol	nt	590	>900	>900	570	>900	>900
fenarimol	1.3	4	310	24	>900	>900	>900
triadimenol	1.4	2	9	10	173	360	200
prochloraz	nt	95	>900	>900	>900	>900	>900
fenpropimorph	nt	6	53	116	14	41	63

Table 5: Fungicide activity of different triazoles in treated and untreated parts of cucumber infected with Sphaerotheca fuliginea, expressed as % disease control. Fungicide concentrations for stem application are 240 mg/l and 60 mg/l and for leaf application 60 mg/l

	stem application			leaf application		
number of leaf	SAN 619 F	propicon-azole	triadi-mefon	SAN 619 F	propicon-azole	triadi-mefon
2	100/25	40/0	55/10	nt	nt	nt
3	100/60	80/0	70/20	0	5	0
4	100/70	95/5	85/35	100	100	95
treated area						
5	0/0	0/0	0/0	100	100	95
6	0/0	0/0	0/0	10	5	0

PREVENTIVE, CURATIVE AND ERADICATIVE ACTIVITY (Figures 2 and 3, Table 6)

When compounds were applied preventively, the duration of fungicide activity varied significantly (Figures 2 and 3). SAN 619 F completely controlled powdery mildew (Uncinula) and especially rust (Puccinia) diseases for at least 12 to 14 days; penconazole did the same but only against powdery mildew. Disease control for a period of about 4 to 8 days was achieved by flusilazole, triadimenol, nuarimol (only for powdery mildew), fenarimol and propiconazole. Other compounds were less active. For curative disease con-

Figure 2 Duration of preventive fungicide activity of different EBI-molecules (at 8 mg/l) against *Uncinula*/grape under greenhouse conditions

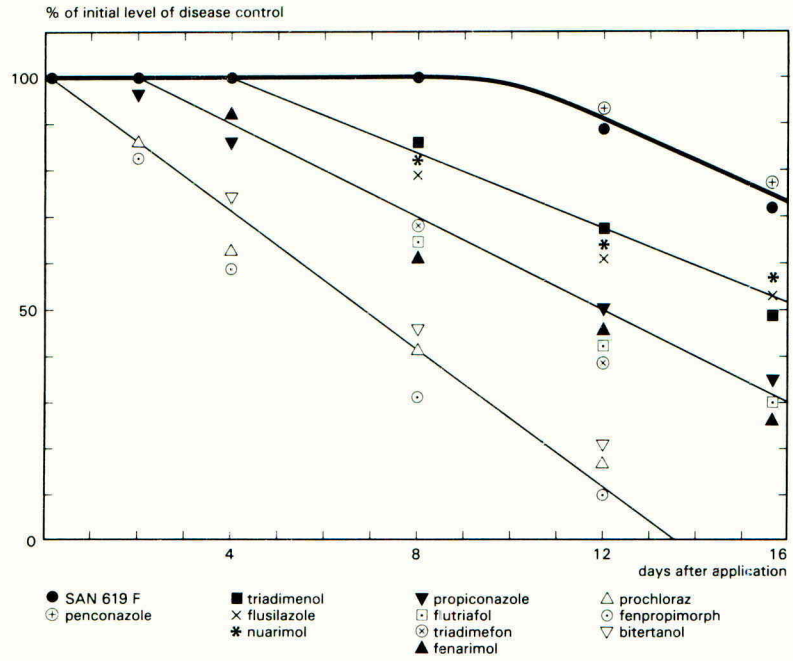
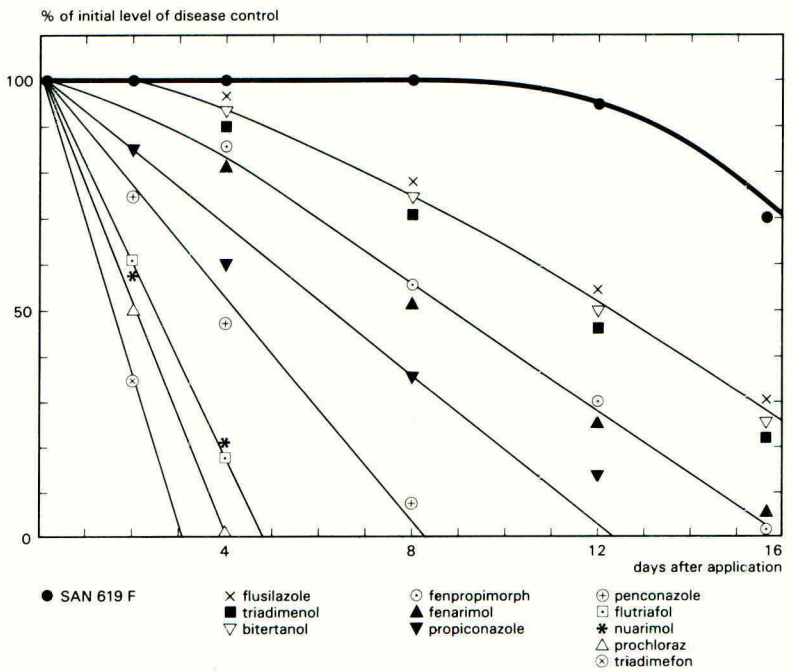


Figure 3 Duration of preventive fungicide activity of different EBI-molecules (at 8 mg/l) against *Puccinia*/snap dragon under greenhouse conditions



trol significantly more material was needed than for preventive control (Table 6). SAN 619 F, propiconazole, triadimenol and fenpropimorph showed interesting levels of curative activity, whereas other compounds had little or no curative activity. Strong eradicative fungicide activity was found with fenpropimorph against powdery mildew; interesting levels of activity were shown by SAN 619 F (against both diseases), propiconazole (against rust) and triadimenol (against mildew).

Table 6: Curative and eradicative fungicide activity of different EBI-molecules against Sphaerotheca fuliginea on cucumber and Uromyces appendiculatus on bean under greenhouse conditions, expressed as EC 90 (mg/l)

test system	SAN 619 F	flusil-azole	propi-con-azole	pen-con-azole	biter-tanol	triadi-menol	pro-chloraz	fen-propi-morph
Sphaerotheca/cucum.								
preventive (0d)	0.6	1.4	0.9	1.5	0.8	2.4	2.4	1.8
curative (3d)	3	3	7	8	151	8	21	32
eradicative (6d)	9	>400	>400	370	>400	32	230	41
Uromyces/bean								
preventive (0d)	0.3	0.7	3.2	2.2	1.5	2.1	13	3.2
curative (3d)	3	10	9	56	>400	10	220	3
eradicative (6d)	47	>400	63	>400	>400	>400	>400	>400

FUNGICIDE ACTIVITY AGAINST DIFFERENT STRAINS OF POWDERY MILDEW (Table 7)

When different field strains of Sphaerotheca (on cucumber) and Erysiphe (on wheat) were exposed to a number of EBI-molecules, the results revealed a decreasing sensitivity to all of the fungicides. The differences between the strains were small for fenpropimorph (factor 2 - 7), as well as for SAN 619 F and triadimenol (factor 1 - 8), whereas intermediate differences (factor 2 - 20) were found for propiconazole, penconazole, fenarimol (RF=9 on cucumber) and strong differences (factor higher than 20) for triadimefon. Since factors below 5 are the result of differences in sensitivity of different strains rather than an indication of resistance, the potential risk of selection for resistance might not be the same when different EBIs are compared. There is positive cross resistance (on different levels) between all tested DMI-fungicides (including fenarimol); but also fenpropimorph was in tendency less active against some strains. Since levels of resistance to EBI-fungicides were not very pronounced (except triadimefon), compared to other fungicide classes, the reported decrease of efficacy of some EBIs in the field may include other reasons like unfavourable application time (low eradicative activity, Table 6) as well as adverse weather conditions (see next paragraph).

INFLUENCE OF TEMPERATURE AND RELATIVE HUMIDITY ON FUNGICIDE ACTIVITY AGAINST POWDERY MILDEW (Table 8)

Fungicide activity clearly depended on temperature and relative humidity. The extent varied with the type of application, the tested host/parasite system and the different molecules. When the systemic activity (translocation from root to leaf or bud to leaf) was tested on cucumber, temperature did not significantly change the fungicide activity against Sphaerotheca. In contrast, all triazoles were 6 to 10 times less effective at 95% rH compared to 60% rH (data not shown, values for 60% rH see Table 4). This observation is indirect proof of fungicide translocation in the transpiration stream which is reduced at high humidity. In the dicot system (cucumber), SAN 619 F, propiconazole and triadimefon were somewhat less active against

Sphaerotheca at higher temperatures, when the humidity was moderate (Table 8); the fungicides were equally active at all temperatures, when the humidity was high. In contrast, all three triazoles were less effective against Erysiphe in the monocot system (barley) at lower temperatures and high humidity, whereas fenpropimorph was more active with decreasing temperature.

Table 7: Preventive fungicide activity of different EBI-molecules against sensitive (s) and "less sensitive" (ls) field strains of Sphaerotheca fuliginea on cucumber and Erysiphe graminis on wheat under greenhouse conditions, expressed as EC 90 (mg/l)

Fungus	strain	SAN 619 F	(pencon-azole) propicon-azole	triadi-mefon	triadi-menol	fen-propi-morph
Spaerotheca fuliginea	60 (s)	0.8	(2)	12	2	18
	51 (ls)	6	(29)	360	9	36
RF[a])	51	8	(15)	30	5	2
Erysiphe graminis	55 (s)	7	10	42	8	39
	109 (ls)	10	204	>900	25	260
	111 (ls)	10	82	>900	nt	132
	107 (ls)	14	23	>900	9	97
RF[a])	109,111	2	8-20	> 20	3	3-7
RF[a])	107	2	2	> 20	1	2

a) RF = resistance factor is calculated by dividing the fungicide activity on the 90 % level (EC 90) of the resistant and sensitive strains; values below 5 are the result of differences in sensitivity of different strains rather than an indication for resistance.

Table 8: Preventive fungicide activity of different EBI-molecules against Spaerotheca fuliginea on cucumber and Erysiphe graminis on barley at different temperature and humidity regimes under greenhouse conditions, expressed as EC 90 (mg/l)

test method	tem-pera-ture (°C)	SAN 619 F		propicon-azole		triadimefon		fenpropimorph	
		60%rH	95%rH	60%rH	95%rH	60%rH	95%rH	60%rH	95%rH
Sphaerotheca/cucumber									
	18	0.4	0.3	1.5	nt	6	2	nt	nt
	22	0.6	0.3	nt	nt	15	2	nt	nt
	26	1.4	0.2	4.0	nt	41	1	nt	nt
Erysiphe/barley									
	10	nt	116	nt	nt	nt	133	nt	2
	15	nt	32	nt	64	nt	28	nt	3
	20	nt	18	nt	48	nt	13	nt	8
	25	nt	13	nt	23	nt	8	nt	11

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POSSIBLE MECHANISMS FOR CONTROL OF *FUSARIUM* SPP. BY *TRICHODERMA HARZIANUM*

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ABSTRACT

A strain of *Trichoderma harzianum* isolated from the rhizosphere of a cotton plant was found to be an effective biological control agent of *Fusarium oxysporum* f. sp. *radicis lycopersici* on tomatoes and of *F. oxysporum* f. sp. *niveum* on watermelons. Application of this antagonist under field conditions as a wheat bran-peat preparation or as a seed coating decreased disease incidence and significantly increased yields of tomatoes and watermelons. Under laboratory conditions, *T. harzianum* did not parasitize colonies of *Fusarium* spp. when tested on agar. However, *T. harzianum* excreted β -1,3-glucanase and chitinase into the medium when grown in liquid media containing laminarin or chitin as a sole carbon source. When cultured on fungal cell wall the hydrolytic enzymatic activity of this strain was lower than that of *T. harzianum* which was antagonistic to *Sclerotium rolfsii* and *Rhizoctonia solani*. Germination of *Fusarium* spp. chlamydospores in soil decreased after the addition of 10^6 conidia of *Trichoderma* per g soil.

INTRODUCTION

Many studies have shown the potential of *Trichoderma* spp. as biocontrol agents of soilborne plant pathogens. However, few data are available showing control of *Fusarium oxysporum* wilt pathogens by *Trichoderma* spp. Marois *et al.* (1981) showed the potential of a multi-fungus conidial suspension including *T. harzianum* in controlling Fusarium crown rot of tomato when applied to a fumigated soil. Locke *et al.* (1985) reported the use of *T. viride* for controlling *F. oxysporum* f. sp. *chrisanthemi* in a steamed soil mix. Sivan & Chet (1986) have recently shown the effectiveness of a newly isolated strain of *T. harzianum* in controlling both Fusarium wilt of cotton and melons and *F. culmorum* on wheat under natural soil conditions.

The direct parasitism of *Trichoderma* on hyphae of other fungi is one of the significant mechanisms which can explain the antagonistic activity of *Trichoderma* spp. (Dennis & Webster, 1971c). Elad *et al.* (1982b) showed that extracellular lytic enzymes (β-1,3-glucanase and chitinase) excreted by *T. harzianum* were responsible for cell wall degradation in *Rhizoctonia solani* and *Sclerotium rolfsii*. However, excretion of antibiotic compounds (Dennis & Webster, 1971a; 1971b) as well as competition (Ahmad & Baker, 1986) may both play major roles in the biological control of soilborne plant pathogens by *Trichoderma* spp.

In the present study we tried to evaluate whether these mechanisms are involved in the biocontrol of *F. oxysporum* wilt pathogens.

MATERIALS AND METHODS

Fungal isolates

An isolate of T. harzianum obtained from a Fusarium-wilted cotton plant and designated T-35 (Sivan & Chet, 1986) served as the biocontrol agent during this study. The hydrolytic enzymatic activity of this isolate on fungal cell wall components was compared to that of T. harzianum TH-203 (Elad et al., 1982b). These isolates were cultured on a synthetic medium (SM; Okon et al., 1973) at 28°C. F. oxysporum f. sp. melonis, F. oxysporum f. sp. vasinfectum, F. oxysporum f. sp. radicis-lycopersici and F. oxysporum f. sp. niveum were cultured on a solidified yeast extract-glucose medium.

Field experiments

Two biological control experiments were carried out in fields naturally infested with F. oxysporum. In the first field experiment, T. harzianum (T-35) was applied for the control of F. oxysporum f. sp. radicis lycopersici, the causal agent of Fusarium crown rot of tomatoes. Tomato field plots (2 beds, 12 x 1.8 m each) were covered with a 0.25 mm thick plastic mulch before planting. T. harzianum was applied as a wheat bran-peat preparation (Sivan et al., 1984) mixed (10% v/v) with the rooting mixture of tomato seedlings (peat and vermiculite 1:1 ; v/v) in the nursery. At the end of the rooting period. Tomato (Lycopersicon esculentum Mill., c.v. 1684-'Naama') seedlings were transplanted in the field, spaced 50 cm apart in one row per bed. The treatments were : a- Control (untreated transplants), b- Transplants treated with T. harzianum, c- Application of 500 kg/ha of methyl bromide (MB), d- Combination of treatments b+c, e- Application of 750 kg/ha of MB, f- Combination of treatments b+e. Treatments composed of 6 replicates. During the season, disease incidence was recorded and expressed as the percent of the population of completely and partially wilted plants. The second biological control experiment involved the control of Fusarium wilt of watermelons induced by F. oxysporum f. sp. niveum. T. harzianum (T-35) was applied as a seed coating (Sivan et al. 1984). Field plots (1 bed, 1.8 x 8 m) were sown with watermelon (Citrullus vulgaris L. c.v. 'Odem') spaced 50 cm apart in one row per bed. The treatments were : a- Control (untreated seeds), b- Seeds treated with conidia of T. harzianum. The treatments consisted of 5 replicates arranged in a randomized block design. During the growing season disease incidence was determined and expressed as the percent of the population of completely wilted plants.

The results of the field experiments were statistically analyzed according to Duncan's multiple range test with a significance level of P=0.05.

Laboratory experiments

Dual culture tests

Tests were conducted according to Dennis and Webster (1971c) on SM, PDA (Difco Laboratories) or on malt extract (Difco). T. harzianum isolates were inoculated 2 days later than Fusarium isolates. Induction of enzymatic activity was performed in liquid cultures. T. harzianum was grown

in liquid SM supplemented with either glucose, laminarin, chitin or fungal cell wall preparation at a concentration of 2 mg/ml. The flasks were inoculated with 0.1 ml of conidial suspension (10^7 conidia/ml). The mycelium was collected by centrifugation and the supernatant was dialyzed against distilled water for 24 h. This material served as a crude enzyme. Cell walls of the tested pathogenic fungi were prepared according to Chet & Huttermann (1980), lyophilized, and ground to fine powder by milling in a coffee mill.

Enzyme assay

β-1,3-Glucanase (EC 3.2.1.39) and chitinase (EC 3.2.1.14) were assayed according to Elad et al. (1982b). Specific activity of glucanase (glucanase Units) was expressed as micromoles of glucose released by 1 milligram of protein per hour. Specific activity of chitinase (chitinase Units) was expressed as micromoles of N- acetylglucosamine released by 1 milligram of protein per hour. The enzymatic activity of these enzymes was also tested using fungal cell wall preparation as a substrate at a concentration of 0.8 mg/ml, and incubating with the crude enzyme for 24 h. Protein determination during enzymatic tests were conducted according to Sedmak & Grossberg (1977).

Chlamydospore germination tests in soil

Enrichment of soil with chlamydospores of F. oxysporum f. sp. melonis and germination tests were carried out according to Sneh et al. (1984).

RESULTS

Field experiments

Tomato transplants treated with T. harzianum (T-35) were better protected against Fusarium crown rot than untreated controls when planted in methyl bromide fumigated or non-fumigated infested plots (Table 1). Application of 750 Kg/ha of MB did not improve disease control when compared with the introduction of the small amount of the fumigant (500 Kg/ha). Moreover, application of the large dose increased the number of the diseased plants. The fumigant alone was more effective than T. harzianum in reducing disease incidence. However, when combined with 500 or 750 kg/ha of MB, T. harzianum significantly improved disease control (as determined by the number of dead plants) by 73.3% and 76.9%, respectively. The effect on incidence of diseased plants was smaller (Table 1). Integration of the antagonist with the reduced dose of the fumigant (500 kg/ha) was more effective than application of either the large or the small dose of MB alone.

T. harzianum was found to be an effective biocontrol agent of F. oxysporum f. sp. niveum. Application of the antagonist as a seed coating resulted in a significant reduction in wilts, and in an increase of 36.6% in the total yield of watermelon (Table 2).

TABLE 1

Integrated control of Fusarium crown rot of tomatoes by T. harzianum and methyl bromide.

Methyl bromide dose (kg/ha)	T. harzianum[1]	Dead Plants[2] (%)	Diseased Plants (%)
0	-	57 a[3]	17a
0	+	40 b	12 ab
500	-	3 c	9 b
500	+	0.8 d	5 b
750	-	6.5 c	17 a
750	+	1.5 d	8 b

(1) T. harzianum was applied as a bran-peat preparation (10%; v/v) mixed with the rooting mixture of tomato seedlings in the nursery.
(2) Plants showing partial wilt symptoms
(3) Numbers in each column followed by the same letter are not signficantly different (P = 0.05) according to Duncan's multiple range test.

TABLE 2

Biological control of F. oxysporum f. sp. niveum on watermelon, by T. harzianum, under field conditions

Treatment	Disease incidence[1] (%)	Total yield (t/ha)
Control	53.5 a	20.5 b
T. harzianum seed coating	22.3 b	28 a

(1) Plants showing complete wilt symptoms.

Laboratory experiments

When T. harzianum was grown in dual cultures against the formae speciales of F. oxysporum: melonis, niveum, vasinfectum and radicis-lycopersici, it did not parasitize the colonies of these pathogens. This lack of parasitism was also observed when cultures were incubated at temperatures ranging from 22 to 33°C.

In liquid cultures of T. harzianum (T-35) containing laminarin or chitin as a sole carbon source, the concentration of extracellular protein ranged between 18 and 25 g/ml compared with 7 g/ml in cultures containing glucose. The amount of extracellular β-1,3-glucanase was twice as high in cultures containing 2 mg/ml laminarin as in cultures containing the same concentration of glucose (Fig. 1) No significant difference (P=0.05) was found between the maximal levels of β-1,3-glucanase and chitinase of T. harzianum isolates (T-35 and TH-203) when cultured on laminarin and chitin, respectively (Fig. 1 & 2).

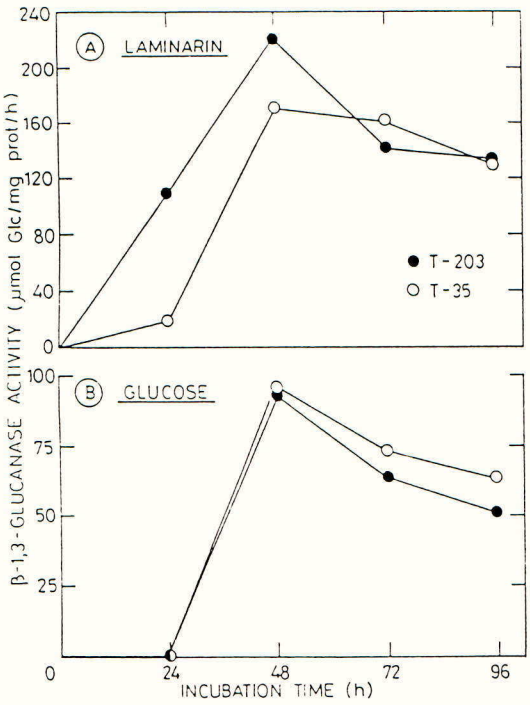

Fig. 1. β-1,3-Glucanase activity of T. harzianum isolates TH-203 and T-35 excreted into liquid SM containing laminarin or glucose (2 mg/ml) as a sole carbon source.

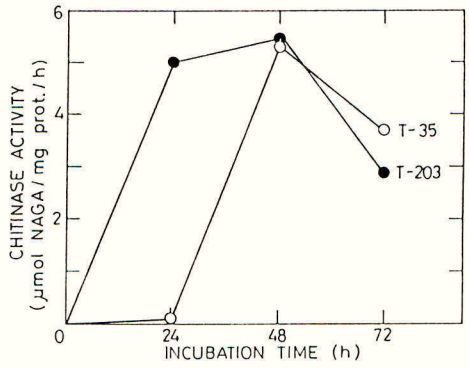

Fig. 2. Chitinase activity of T. harzianum isolates TH-203 and T-35 excreted into liquid SM containing chitin (2 mg/ml) as a sole carbon source.

When T. harzianum isolates were grown on cell wall preparation of F. oxysporum f. sp. melonis, TH-203 excreted a higher level of β-1,3-glucanase and chitinase: 191.3 and 42.7 Units, compared with 156 and 21.4 Units produced by T-35, respectively. When incubated with cell wall preparation of F. oxysporum f. sp. melonis, crude enzymes of both isolates of T. harzianum released a similar amount of glucose, 0.46 and 0.32 Units by TH-203 and T-35, respectively. However, TH-203 released more N-acetylglucosamine (5.04 Units) than did T-35 (0.5 Units).

Chlamydospore germination tests in soil

Germination rate of chlamydospores of F. oxysporum f. sp. melonis in soil ranged from 32 to 37%. Amendment with solutions containing glucose and asparagine (5:1; w/w) increased the percent of germinated chlamydospores. Maximal germination (up to 46%) was obtained in soil amended with 0.1 mg of glucose and 0.02 mg of asparagine per g soil (Fig. 3).

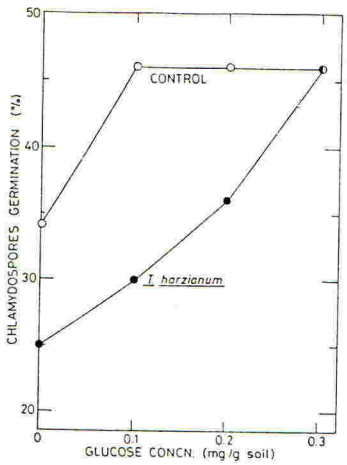

Fig. 3. Chlamydospore germination of F. oxysporum f. sp. melonis as affected by conidia of T. harzianum (T-35; 10^6/gsoil) in soil amended with glucose and asparagine (5:1; w/w).

Pregerminated conidia of T. harzianum (T-35) inhibited chlamydospore germination when applied to a soil amended with glucose and asparagine. Maximal inhibition was observed in samples amended with 0.1 mg of glucose and 0.02 mg of asparagine per g soil (Fig. 3). However, higher concentrations of nutrients minimized the inhibitory effect of T. harzianum.

DISCUSSION

This study has shown the potential of T. harzianum as a biocontrol agent of two Fusarium wilt pathogens, F. oxysporum f. sp. radicis - lycopersici and F. oxysporum f. sp. niveum. In the past, application of the same isolate of T. harzianum under greenhouse conditions resulted in significant control of F. oxysporum on cotton and melon and of F. culmorum on wheat (Sivan & Chet, 1986).

When this antagonist was integrated with 500 or 750 kg/ha of methyl bromide in a tomato field naturally infested with Fusarium crown rot, the disease control was improved. The higher dose of the fumigant was found to be less active than the lower dose (500 kg/ha). This may be due to a higher reinfestation by the pathogen, of plots fumigated with the larger dose of the fumigant. Soil disinfestation induces a biological vacuum, hence, it is possible to assume that air-borne spore dispersal and recolonization of fumigated soil by this pathogen (Rowe, et al., 1977) were more effective in plots treated with the higher amount of MB.

Dennis & Webster (1971c) reported the successful parasitism of F. oxysporum by several isolates of T. harzianum. Similarly, Elad et al. (1982a) showed the hyperparasitism of S. rolfsii and R. solani by T. harzianum. However, in this study, the efficiency of T. harzianum in the biocontrol of Fusarium wilt pathogens was not correlated with the results obtained from the dual culture tests. Elad et al. (1982b) demonstrated the enzymatic potential of T. harzianum (TH-203) to utilize cell wall constituents of R. solani and S. rolfsii. Therefore, one of the major questions is whether or not our antagonistic isolate has this enzymatic potential to degrade fungal cell wall components. Comparison of the lytic activity of our isolate with that of TH-203 revealed that when cultured on laminarin or chitin as a sole carbon source, the level of β-1,3-glucanase and chitinase excreted into the medium was similar. However, when grown on F. oxysporum f. sp. melonis cell wall preparation, the lytic activity of TH-203 was higher than that of T-35. Similarly, crude enzyme of TH-203 released more N-acetylglucosamine from Fusarium cell wall preparation than did that of T-35. It is not clear whether the lower lytic capacity of T-35 is the only reason for the lack of parasitism in dual culture tests, since lack of recognition between fungi might prevent hyphal interactions (Barak et al., 1985).

Baker (1981) reported that Fusarium pathogens are very sensitive to lack of carbon and nitrogen sources in soil. Scher & Baker (1982) demonstrated the competition for Fe^{3+} between F. oxysporum and Pseudomonas putida. Similarly, Pseudomonas isolates inhibited chlamydospore germination of F. oxysporum f. sp. cucumerinum in soil amended with glucose and asparagine (Sneh et al., 1984). In this study, we have shown inhibition of chlamydospore germination of F. oxysporum f. sp. melonis by pregerminated conidia of T. harzianum in soil enriched with chlamydospores and amended with nutrient solutions of glucose and asparagine. The inhibitory effect was eliminated in the presence of an excess of nutrients, indicating that competition may play a role in the antagonistic activity of T. harzianum against Fusarium wilt pathogens.

ACKNOWLEDGEMENTS

This research was partially supported by a grant from the United States - Israel Binational Agricultural Research and Development Fund (BARD), and by Makhteshim Co., Beer Sheva, Israel). We thank O. Ucko and S. Biton from the Extension Service, The Ministry of Agriculture, Dr. A. Grinberger from the Netiv-Hagdud Experimental Station, Mr. Y. Shitrit of The Hebrew University for technical assistance, and Bromide Compounds, Beer Sheva, Israel for supplying and applying the fumigant.

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COMPARATIVE LABORATORY STUDY OF PHOSMETHYLAN

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ABSTRACT

The effect of phosmethylan and the influence of temperature and site of application on the toxicity were examined using the bioassay method. Similar effects of the product were observed when used either as contact or as stomach poison. Neither systemic, repellent nor vapour effect of the material was found, but its antifeedent effect is considerable. Treating the head, the thorax and the abdomen topically the most effective treatment was found to be on the thorax. A positive temperature coefficient was found for phosmethylan.

INTRODUCTION

Phosmethylan is an a.i. discovered in 1979 at the Research Institute for Heavy Chemical Industries and registered by the ISO in 1985. The agent was developed and distributed with the trade mark Nevifosz 50 EC. It was introduced to the international public at the ICPP in Brighton in 1983 (Sagi et al., 1983). The results reported there referred mainly to large scale investigations.

In this paper, bioassay examinations have been carried out to determine the mode of action of phosmethylan and some of the factors influencing its effect. Contact, stomach poison, systemic, vapour and antifeedent effects were examined and the effect of temperature and of the site of application on the biological activity were also studied.

MATERIALS AND METHODS

To observe the mode of action the following bioassay tests have been performed.

The separate contact and stomach poison effects were studied on turnip sawfly larvae (Athalia rosae), treating leaf discs of the same size, using a micropipette. The contact effect was tested by treating "non host plant" mulberry tree (Morus alba) leaves, while the stomach poison effect was studied on winter oilseed rape (Brassica napus) using the leaf sandwich method. To observe the simultaneous contact and stomach poison effects winter oilseed rape leaf discs were used for feeding.

The effect of the compound on the feeding of insects was examined on mustard beetle (Phaedon cochleraria), on Chinese cabbage (Brassica chinensis) and on diamondback moth (Plutella maculipennis) on white mustard (Sinapis alba) test plants. The effect of time on the number of feeding larvae was observed and the surface area of the leaves eaten was measured with a leaf area meter (Licor Li-300).

When the degree of toxicity and the role of site and temperature of the treatments were tested, 3-5 days old female adults of WHO/SRS house

fly were used. 1 µl phosmethylan with a purity of 99% was dissolved in acetone and this solution was used for treatment with a microapplicator of type LV.65. 5–7 dose values were applied for each variation, with 3 replications per dose, with 20 flies per replication. The tests were carried out at a temperature of 24 ± 1°C and relative humidity of 65 ± 5%. The data obtained were evaluated by probit analysis.

To find a connection between the degree of toxicity and the place of treatment, the head, the dorsal part of the thorax and the ventral part of the abdomen were treated. Tests were performed at temperatures of 15, 20, 25 and 30°C to discover the relationship between toxicity and temperature.

RESULTS

Apoplastic, symplastic and translaminar translocation of the phosmethylan, penetration from the one side of the leaf to the other side or vapour and repellent effects were not proved with biological indicator organisms.

The results of the separate and simultaneous contact and stomach poison tests are given in Table 1. In the case of high dose values (5.6 µg/cm^2) activities equalling or approaching 100% were found for all of the tests, while with lower doses (2.8 µg/cm^2) the mortality caused by the separate contact and stomach poison effects is less than that of the simultaneous action.

TABLE 1

Test of the separate and simultaneous contact and stomach poison effects of phosmethylan on turnip sawfly larvae (L_3)

Method of test	Dose a.i. (µg/cm^2)	Mortality after 24 hours (%)
Contact	5.6	100
	2.8	82
Stomach poison	5.6	97
	2.8	78
Simultaneous contact and stomach poison effect	5.6	100
	2.8	100

The feeding after treatment is given in Fig. 1 for mustard beetle and in Fig. 2. for diamondback moth larvae. The results of the consumption of leaf area are given in Table 2, compared with untreated leaf as a blank.

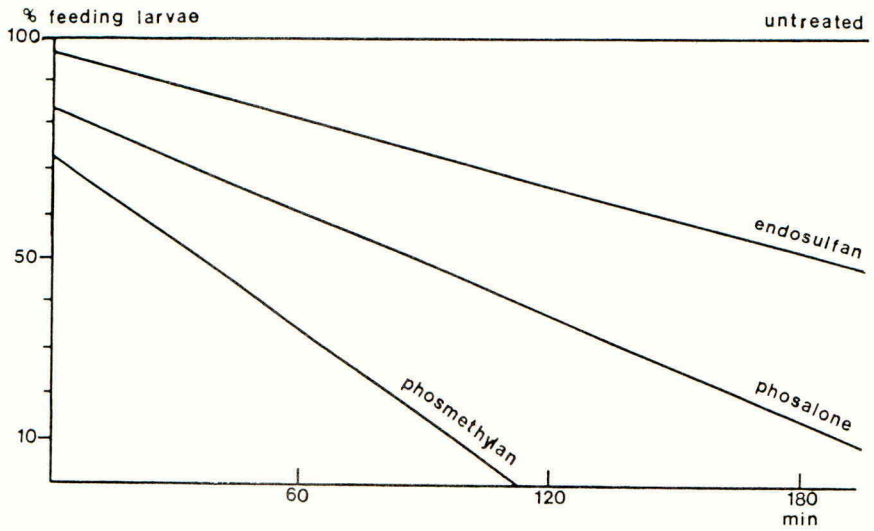

Fig. 1. Food consumption of P. cochlerariae (L_4) after treating the leaves with 125 mg a.i./l. Ratio of feeding larvae plotted against time, %.

phosmethylan Y'= 73.83-0.43X b=0.43±0.06 r=-0.91
endosulfan Y'=104.94-0.31X b=0.31±0.03 r=-0.96
phosalone Y'= 84.45-0.43X b=0.43±0.04 r=-0.96
untreated Y'= 96.00-0.1 X b=0.1 ±0.01 r=-0.7

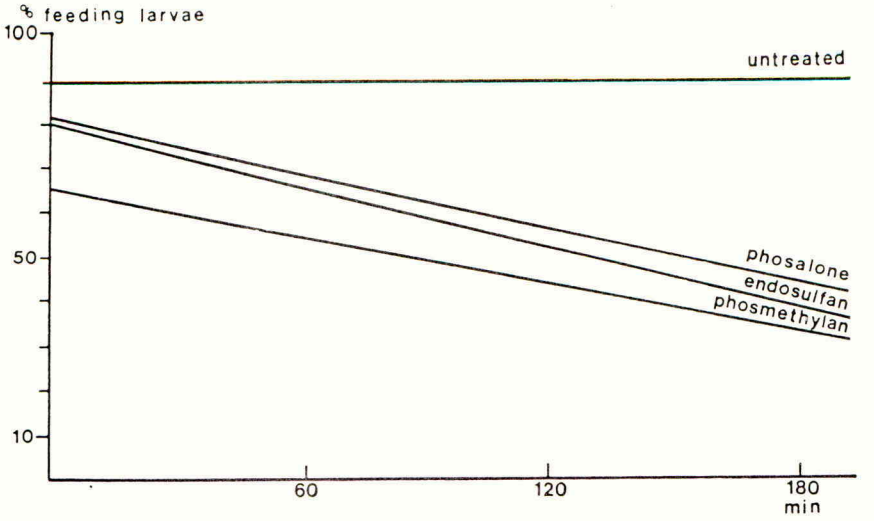

Fig. 2. Food consumption P. maculipennis (L_3) after treating the leaves with 125 mg a.i./l. Ratio of feeding larvae plotted against time, %.

phosmethylan Y'= 71.86-0.25X b=-0.25±0.66 r=-0.8
endosulfan Y'= 81.62-0.26X b=-0.26±0.04 r=-0.91
phosalone Y'= 81.33-0.24X b=-0.24±0.04 r=-0.89
untreated Y'= 89.76-0.1 X b=-0.1 ±0.01 r=-0.8

TABLE 2

Feeding of mustard beetle L_4 (PHA) and diamondback moth L_3 (PLU) larvae after a dipping with 125 mg a.i./l for 185 minutes of observation.

Treatment	Food consumption as a % of the untreated	
	PLU	PHA
Phosmethylan	53.4	24.8
Endosulfan	48.0	91.2
Phosalone	67.5	77.9
Untreated	100.0	100.0

It is known from the literature that in the case of topical application, the degree of toxicity of insecticides depends on the site of application (Hewlett and Gostick, 1963; Ahmed and Gardiner, 1968). Similar differences in dose-effect values were found for phosmethylan applied on the three different parts of the insects. In the first series of experiments (Table 3) the LD_{50} value measured on the thorax is significantly lower than that on the head and is not significantly lower than on the dorsal. In the second series of experiments the phosmethylan proved to be significantly more effective on the thorax than either on the head or on the dorsal part.

TABLE 3

Dose-response data for phosmethylan topically applied to the various body parts of adult Musca domestica L.

No. of test	Site of application	LD_{50} ($\mu g/\varphi$)	Fiducial-limits (95%)		Slope	±	SE
			Lower	Upper			
1	Head	0.98	0.87	1.09	2.91	±	0.42
	Thorax	0.54	0.49	0.59	4.29	±	0.73
	Abdomen	0.61	0.56	0.67	4.18	±	0.78
2	Head	1.88	1.54	2.29	1.96	±	0.42
	Thorax	1.09	0.91	1.30	2.68	±	0.50
	Abdomen	1.47	1.30	1.66	3.78	±	0.69

The knowledge of the relationship between toxicity and temperature is crucial from the point of view of practical plant protection. From the results of phosmethylan tests performed with house flies the conclusion can be drawn that this material has a positive temperature coefficient (Fig. 3). This observation is confirmed by the results of both series of experiments, though significant differences were found only in the first series of experiments (Table 4). There is no considerable difference between the results obtained at 20°C and 25°C. On the other hand the toxicity of the phosmethylan is significantly lower at 15°C and significantly higher at 30°C than at the temperatures listed above. The doubled value of toxicity at 30°C compared with that at 25°C is especially remarkable.

TABLE 4

Dose-response data for phosmethylan topically applied to the WHO/SRS housefly at different temperature in two series of experiments.

No. of test	Temperature (°C)	LD_{50} (µg/♀)	Fiducial-limits (95%) Lower	Upper	Slope	±	SE
1	15	2.01	1.79	2.25	2.94	±	0.44
	20	1.48	1.35	1.64	3.75	±	0.59
	25	1.46	1.30	1.63	3.04	±	0.51
	30	0.69	0.62	0.78	2.69	±	0.34
2	15	1.92	1.70	2.18	2.55	±	0.39
	20	1.76	1.58	1.97	2.93	±	0.42
	25	1.55	1.40	1.73	3.07	±	0.43
	30	1.34	1.22	1.48	3.68	±	0.52

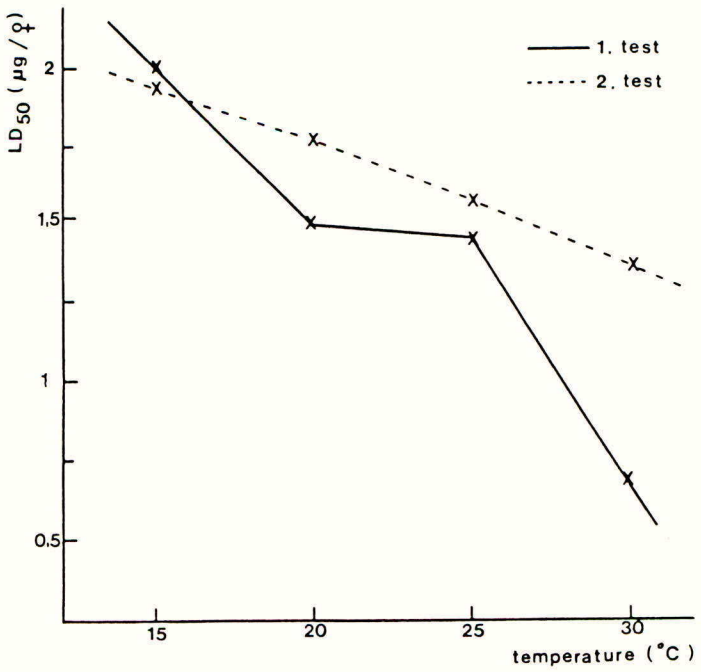

Fig. 3. LD_{50} values for phosmethylan plotted against different temperature.

DISCUSSION

From the results of the tests carried out to discover the mode of action of the phosmethylan, the conclusion can be drawn that the compound has contact and stomach poison effects. Systemic, vapour and repellent effects were not proved with test organisms. Nevertheless the food consumption of mustard beetle and diamondback moth was considerably hindered.

By applying topical treatments on different parts of the body of flies it was observed that the best results can be obtained, if the treatment is performed on the thorax.

The toxicity of insecticides is influenced by several factors, one of which is temperature. Some insecticides have higher toxicity at higher temperature (organophosphates, carbamates), while others have lower toxicity at higher temperature (DDT, pyrethroids). The positive temperature dependence of the insecticide is demonstrated by the tests carried out with phosmethylan on house flies. In one test the efficiency at 30°C is more than twice that at either 20°C or 25°C.

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LIPID PEROXIDATION, A CONSEQUENCE OF THE MECHANISM OF ACTION OF AROMATIC HYDROCARBON AND DICARBOXIMIDE FUNGICIDES AND A SIDE EFFECT IN DMI FUNGICIDES [1)]

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ABSTRACT

The mechanism of action of aromatic hydrocarbon fungicides (AHF) and dicarboximide fungicides (DF) has been obscure in the past years. Recent investigations in our laboratory demonstrate that these fungicides induce a lipid peroxidation in sensitive fungi. The cause of this pathological and lethal event is assumed to be a specific interaction of the fungicides with flavin containing enzymes. Although several flavin enzymes are influenced in various directions and to various degrees by AHF and DF, a "cytochrome c reductase" seems to have a key role because it is inhibited by all of these fungicides. This inhibition leads, probably by a radical mechanism, to lipid peroxidation which can be avoided by an addition of α-tocopherol acetate. In Mucor mucedo all investigated DMI fungicides, such as fenarimol, etaconazole, imidazole, prochloraz and others, but not tridemorph or dichlofluanide, also induce, at high concentrations, a lipid peroxidation well correlated with a growth inhibition which can in most cases be nullified by addition of α-tocopherol. No cross resistance exists in M. mucedo between DMI and AHF or DF.

INTRODUCTION

The mechanism of action of the main groups of fungicides has largely been elucidated in the past decades. This has not been the case for aromatic hydrocarbon and dicarboximide fungicides although the former group has already been used for several decades. According to Leroux & Fritz (1984) DF provoke numerous pathological effects in fungal cells, effects which are very similar to those produced by AHF. In general, both groups of fungicides surprisingly exhibit cross resistance to resistant fungal strains. This could be based on a common mechanism of action, but some exceptions have been described (Beever & Byrde, 1982).

Several effects of these fungicides have been described, such as inhibition of DNA or protein synthesis, effects on hereditary processes, lipid synthesis membranes and others. However, the results have been very contradictory and do not offer a very convincing explanation of the mechanism of action of these fungicides (cf. Leroux & Fritz, 1984). Until the mechanism of action is elucidated nothing can be said regarding the mechanism of resistance or cross resistance.

[1)]Investigations on DF fungicides were supported by BASF AG (Limburgerhof). We thank Drs Mangold, Pommer and Lorenz for their stimulating interest.

DMI fungicides interfere with oxidative sterol demethylation at the 14-C position by inhibiting a cytochrome P 450 monoxygenase (Buchenauer, 1986, in the press). Using Mucor mucedo as fungus we found, surprisingly, a strong lipid peroxidation under the influence of DMI fungicides in a good correlation to growth inhibition, which suggests a second mechanism of action of these compounds.

MATERIALS AND METHODS

In our investigations we used wild strains of Mucor mucedo or Botrytis cinerea as well as chloroneb resistant strains of M. mucedo or dicarboximide resistant strains of B. cinerea. Young mycelia were produced by mass spore inoculation into a medium according to Coursen & Sisler (1960).

Lipid peroxidation was measured using thiobarbituric acid according to the method of King et al. (1975). NADPH dependent cytochrome c reductase activity was detected by the method of Pederson et al. (1973). NADPH oxidase activity was usually determined by decrease of extinction at 340 nm.

RESULTS

Previous investigations with chloroneb (Lyr & Werner, 1982) or etridiazole (Radzuhn & Lyr, 1984) revealed that an unconventional mechanism of action is in operation in these fungicide groups. We concluded that induced lipid peroxidation was the reason for the many events described in the literature (Lyr et al., 1984; Edlich & Lyr, 1984; Lyr, 1986, in the press; Edlich & Lyr, 1986, in the press). A compound such as chloroneb which is chemically quite inert cannot per se induce lipid peroxidation but could only be able to induce it by interaction with suitable targets. The same is true for dicarboximide fungicides. By their structures, AHF or dicarboximide fungicides could interact with cytochrome P 450 monoxygenase or a similar enzyme, for example a flavin monoxygenase. Indeed chloroneb does interact with cytochrome P 450 (rat liver microsomes) giving a type I substrate binding spectrum (unpublished).

However, further investigations revealed that P 450 can be excluded as the target enzyme in M. mucedo and B. cinerea, because the lipid peroxidation caused by the fungicide was not inhibited by KCN or KSCN, which inactivate cytochrome P 450. In M. mucedo no cytochrome P 450 enzyme has yet been demonstrated. Thus, other target enzymes are more probable, such as flavin monoxygenases. Like cytochrome P 450 monoxygenase, they are known to produce free radicals under certain circumstances. This may result in a lipid peroxidation of surrounding phospholipids (Ziegler et al., 1980).

Chloroneb, as well as other compounds of this group, strongly inhibits the cytochrome c reductase activity of a crude preparation from sensitive M. mucedo (Table 1). Simultaneously the NADPH oxidation is enhanced drastically.

TABLE 1

Inhibition of a NADPH dependent cytochrome c reductase activity from Mucor mucedo homogenates by addition of chloroneb and vinclozolin

Compound	Concn (M)	Cytochrome c reduction[1)]
-	-	100
Chloroneb	5×10^{-7}	87
	10^{-7}	64
	5×10^{-6}	22
	10^{-6}	0
Vinclozolin	5×10^{-7}	95
	10^{-7}	82
	5×10^{-6}	69
	10^{-6}	32

[1)]Activity of reduced cytochrome c is expressed as a percentage of an untreated control.

Other flavin enzymes are also affected to varying degrees and in various ways by fungicides of both groups (Table 2). This means that effects on enzymes of this type can give rise to side effects which may be of physiological significance, but there is no strict correlation of effects on the enzymes with the growth inhibiting effect of the fungicides. Therefore it is improbable that the interactions with flavin enzymes, such as glucose oxidase, xanthine oxidase, NADPH oxidase, amino acid oxidase or glutathione reductase are as closely related to the primary mechanism of action as the inhibition of a "cytochrome c reductase" (the physiological role of which is still obscure).

TABLE 2

The effect of various fungicides on the activity of some flavin containing enzymes[1)]

Compound	Concn (M)	DAAO	GOD	XOD	GR
-	-	100	100	100	100
Chloroneb	5×10^{-4}	66	192	0	62
Etridiazole	5×10^{-4}	98	104	97	94
Quintozine	5×10^{-4}	58	143	26	147
Procymidone	5×10^{-4}	48	167	90	0
Tolclophos-methyl	5×10^{-4}	54	186	93	112
Vinclozolin	5×10^{-4}	22	122	78	86

1)Enzyme activities are expressed as a percentage of an untreated control.

Abbreviations: DAAO - D-aminoacid oxidase; GOD - Glucose oxidase; XOD - Xanthine oxidase; GR - Glutathione reductase.

It should be mentioned, however, that glucose (and xylose)oxidase and their stimulation by DF play an important role in the infection mechanism of B. cinerea and probably some other fungi (Edlich & Lyr, 1987, in the press).

The importance of the inhibitory properties of AHF and DF is strongly supported by the fact that α-tocopherol acetate alleviates growth inhibition and lipid peroxidation. This is demonstrated in Table 3 for some AHF, DF and DMI fungicides. The same is true for other members of these groups of fungicides not mentioned here. It is remarkable that other sterol biosynthesis inhibitors such as tridemorph, or unrelated fungicides such as dichlofluanid, do not induce lipid peroxidation (Lyr, 1987, in the press).

TABLE 3

The effect of α-tocopherol on growth inhibition and lipid peroxidation induced by fungicides of AHF, DF or DMI-type in Mucor mucedo.

Compound	Concn (M)	Growth inhibition	Lipid peroxide content[1)]
-	-	0	100
α-Tocopherol (T)	5×10^{-5}	6	108
Chloroneb	3×10^{-6}	56	178
Chloroneb + T		23	124
Fenarimol	5×10^{-5}	70	230
Fenarimol + T		13	90
Quintozine	8×10^{-6}	66	207
Quintozine + T		38	149
Prochloraz	5×10^{-5}	40	135
Prochloraz + T		60	133
Triforine	5×10^{-5}	36	140
Triforine + T		0	80
Vinclozolin	2×10^{-5}	36	144
Vinclozolin + T		12	108

1)Data derived from various test series and expressed as a percentage of an untreated control.

α-Tocopherol itself in higher concentrations can exhibit some toxicity and induce lipid peroxidation because of its redox character, but after insertion into fungal membranes it protects the fungus against lipid peroxidation and cell destruction because it then acts as a radical scavenger and prevents further spread of peroxidation in lipid containing structures.

Whereas AHF and dicarboximides are well known to exhibit cross resistance in restant strains (Leroux & Fritz, 1984), no indication for cross resistance to DMI fungicides exists. Because of the similar effects regarding the induction of lipid peroxidation in M. mucedo, we tested S- and R_{chl}-strains of this fungus with respect to cross resistance against DMI fungicides. The results demonstrate (Table 4) no cross resistance exists between AHF and DMIs.

TABLE 4

Growth inhibition and lipid peroxidation in a S- and R-strain (chloroneb resistant) of Mucor mucedo under the influence of DMI fungicides related to controls.

Fungicides	Concn	S-strain		R-strain	
		growth inhibn	lipid peroxide	growth inhibn	lipid peroxide
	mg/l	%	%	%	%
Fenarimol	10	16	123	38	160
Triforine	100	11	149	5	151
Triadimefon	100	62	169	23	169
Prochloraz	50	79	210	100	-

DISCUSSION

As this brief description may suggest, induction of lipid peroxidation is a new mode of action in some groups of fungi. It is the main mode of action of AHF and dicarboximide fungicides and is responsible for their fungitoxic effect.

Probably it is a secondary mechanism of action in DMI fungicides. Its practical significance is still under investigation. Although inhibition of the oxidative demethylation of sterols by DMIs is undoubtedly their primary mechanism of action as many investigators have convincingly demonstrated (Buchenauer, 1986, in the press), some hints exist in the literature of a secondary mechanism which is in operation at higher concentrations. M. mucedo seems to be a suitable test species in which to search for this because of its low sensitivity to demethylation inhibition.

Tables 3 and 4 indicate that, although the final result in the cell, i.e. lipid peroxidation of membrane phospholipids, is the same in the case of DMI treatment as for treatment with other fungicides, the primary events leading to this effect seem to be different because no cross resistance exists between DMI and the other fungicide groups. This means that different target enzymes are probably involved.

At present it is still uncertain whether DMI fungicides exclusively interact with the haem part of cytochrome P 450 monooxygenase or can also interact with the cytochrome c reductase part of this enzyme. Experiments aimed at elucidating this are running in our laboratory. It is also possible that DMI fungicides interact with still other flavin enzymes. That this mechanism is of vital importance at least for a fungus such as M. mucedo is demonstrated by the fact that growth inhibition and lipid peroxidation can be diminished to the same extent by addition of the radical scavenger α-tocopherol (with the as yet unexplained exception of prochloraz). The eluddidation of this area could provide deeper understanding of the resistance phenomenon in DMI.

The same of course is true for AHF and dicarboximides. The occurrence of resistance to these is more widespread than is the case with DMI fungicides perhaps because the basis of resistance is less complex. At present we can only say that degradation or reduced uptake of the fungicides can be excluded as the reason for resistance. Other possibilities are under investigation.

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