Biocontrol Science and Technology (1992) 2, 49–58

Influence of Fungicides and Insecticides on the Entomogenous Fungus *Metarhizium anisopliae*, a Pathogen of the Vine Weevil, *Otiorhynchus sulcatus*

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(*Received for publication 31 July 1991*)

In the laboratory, the fungicides chlorothalonil and zineb prevented germination of Metarhizium anisopliae *conidia when incorporated into Sabouraud dextrose agar (SDA) at the commercial concentration (based on the manufacturers' recommended rates for horticultural crops). Twelve other fungicides and six insecticides had no effect on spore germination when applied at the same rate. Mycelial growth of* M. anisopliae *on SDA plates containing the recommended rate of all the pesticides (except propamocarb) was reduced compared with SDA alone. Two fungicides, benomyl and carbendazim, totally inhibited growth at 0.1 times the recommended rate. Growth was also completely prevented by the fungicides etridiazole, triforine and zineb, and the insecticides dichlorvos and hostathion, at 10 times the recommended rate. In a glasshouse experiment, a prophylactic drench of* M. anisopliae *conidia reduced vine weevil* (Otiorhynchus sulcatus) *populations on* Impatiens *plants by 88%. This level of control was not significantly reduced by subsequent application (7 days after egg infestation) of any of the pesticides at the recommended concentration. Larval control in pots treated with* M. anisopliae *plus any one of the 12 fungicides and four insecticides examined, ranged from 82% to 98%. The insecticide diazinon applied alone reduced larval numbers by 100%. Two other insecticides, dichlorvos and cypermethrin, and the fungicide pyrazaphos, also reduced weevil populations by over 50%. These experiments demonstrate the limitations of laboratory based* in vitro *screening programmes for assessing the chemical compatibility of* M. anisopliae.

Keywords: *entomogenous fungus,* Metarhizium anisopliae, Otiorhynchus sulcatus, *pesticide compatibility, biological control, conidial germination, mycelial growth*

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INTRODUCTION

Entomogenous fungi are important natural regulators of insect populations and have potential as mycoinsecticides (Gillespie & Moorhouse, 1989). However, a number of recent studies suggest that they may be adversely affected by pesticides. Nanne & Radcliffe (1971) noted reduced levels of aphid infection by *Entomophthora* on potato plots sprayed with Bordeaux mixture, captafol or mancozeb, and Johnson *et al.* (1976) found that control of *Anticarsia gemmatalis* by *Nomuraea rileyi* was delayed by pesticides. The sensitivity of natural control systems to pesticides indicates the need to consider the chemical compatibility of potential microbial control agents.

The entomogenous fungus, *Metarhizium anisopliae*, has shown considerable promise as a microbial control agent of the vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) (Moorhouse, 1990). This pest causes serious damage to glasshouse pot plants, hardy ornamental nursery stock and soft fruit. Commercial application of *M. anisopliae* will necessitate integration with other crop protection products and consequently it is necessary to evaluate its compatibility with pesticides.

The influence of pesticides on the germination and growth of economically important species of entomopathogenic fungi, such as *Beauveria bassiana* (Olmert & Kenneth, 1974), *Entomophthora obscura* (Öncüer & Latteur, 1979), *M. anisopliae* (Tedders, 1981) and *Verticillium lecanii* (Hall, 1981) has been examined extensively on agar. However, there are no reports of *in vivo* studies with *M. anisopliae* and relatively few reports on other entomogenous fungal species. Some of the limitations of *in vitro* compatibility studies were demonstrated by the work of Wilding & Brobyn (1980). The germination and growth of *Erynia neoaphidis* (described as *Entomophthora aphidis*) was inhibited by the recommended rate of all the fungicides examined, but aphid infection was not reduced by pesticide application 22 or 72 h after inoculation. *In vitro* studies therefore have limited value unless they can be compared with similar *in vivo* experiments. Furthermore, a lack of *in vitro* inhibition is no indication of compatibility because some compounds, such as propamocarb, can have greater activity in soil than in agar (Rapp & Richter, 1982).

The present study was initiated to examine the value of *in vitro* pesticide compatibility studies for predicting the performance of *M. anisopliae* against *O. sulcatus* in a commercial situation.

MATERIALS AND METHODS

The strain of *M. anisopliae* used in these experiments (275-86) was originally isolated from codling moth, *Cydia pomonella*, in Western Germany and it was selected because it is highly virulent towards *O. sulcatus* larvae (Moorhouse, 1990). Conidia were produced on Sabouraud dextrose agar (SDA; Oxoid Ltd, Basingstoke) in Petri plates (9 cm in diameter) which were inoculated with conidia and incubated for 10 days at 23°C.

The 14 fungicides and six insecticides (Table 1) used in this study were selected from a range of commonly used horticultural pesticides. The application rates in most cases were obtained from the manufacturer's recommendations for glasshouse crops in product literature. Where these were not available, estimates were based on dosages recommended for similar crops.

In vitro Tests on Germination and Growth

SDA was mixed with deionized water (65 g l^{-1}) and autoclaved in 250 ml Erlenmeyer flasks. The sterile agar was cooled in a water bath to approximately 55°C before addition of pesticides. Three flasks were prepared for each pesticide using 0.1, 1 and 10 times the recommended rates. Each pesticide medium was then either poured into 9 cm Petri dishes (approximately 20 ml per plate) or pipetted onto a clean glass microscope slide in three discrete pools (recommended rate only) using a sterile Pasteur pipette. Each slide was then placed on three moist Whatman no. 1 filter papers in a Petri dish.

TABLE 1. Pesticides used in the laboratory studies

Active ingredient	Product	% Active ingredient	Formulation[a]	Rate of use g or ml l^{-1}
(a) Fungicides				
Benomyl	Benlate	50	w.p.	0.50
Bupirimate	Nimrod	25	e.c.	0.70
Carbendazim	Bavistin	50	w.p.	1.10
Chlorothalonil	Repulse	50	s.c.	2.16
Etridiazole	Aaterra	35	w.p.	1.50
Fenarimol	Rubigan	12	w.p.	0.18
Furalaxyl	Fongarid	25	w.p.	0.30
Iprodione	Rovral	50	w.p.	1.00
Propamocarb	Filex	72.2	e.c.	1.50
Pyrazaphos	Afugan	29.5	e.c.	1.00
Quinomethionate	Morestan	25	w.p.	1.00
Triforine	Saprol	19	e.c.	1.25
Tolclofos-methyl	Basilex	50	w.p.	2.00
Zineb	Zineb	70	w.p.	2.00
(b) Insecticides				
Aldrin	Aldrin	30	w.p.	1.60
Cypermethrin	Ambush	10	e.c.	1.40
Diazinon	Diazitol	16	e.c.	1.00
Dichlorvos	Nuvan	50	e.c.	1.00
Hostathion	Hostaquick	50	e.c.	0.75
Pirimicarb	Pirimor	50	w.p.	0.375

[a](e.c.) Emulsifiable concentrate; (s.c.) soluble concentrate; (w.p.) wettable powder.

A conidial suspension of *M. anisopliae* was prepared by suspending a loopful of spores in a universal bottle containing 0.05% sterile Triton X-100 (BDH Chemicals Ltd, Poole). The concentration was determined using an improved Neubauer haemocytometer and adjusted to 10^6 conidia ml^{-1}. This suspension was sonicated (amplitude of 10 μm) for approximately 10 s to break up the spore clumps and then 0.01 ml were spread on the three agar pools on each microscope slide. The slides were dried in a laminar flow cabinet for 5 min before being replaced in the Petri dishes which were then sealed in plastic bags. After 24 h incubation at 25°C, germination was determined on SDA alone (control) and on each pesticide medium by observing 100 conidia on each agar pool using phase-contrast microscopy.

An SDA plate was prepared by evenly spreading 0.05 ml of the 10^6 conidia per ml suspension (prepared above) over the agar surface. Agar plugs were removed after 24 h incubation at 25°C using a sterile 5 mm diameter cork borer. One *M. anisopliae* plug was inverted into a matching hole, previously cut in the centre of each SDA or pesticide media plate, using a dissecting needle. Three replicate plates were prepared for each pesticide concentration and six replicate plates containing only SDA served as controls. Plates from each treatment were incubated separately at 25°C inside plastic bags to reduce any possible effects from volatile pesticides. Mycelial growth was assessed every 3 or 4 days by measuring the colony diameter on each plate (two measurements made at right angles). The plates were examined five times over a 17-day period and the radial extension rates were calculated by regression analysis. These rates were then compared by analysis of variance (ANOVA).

Glasshouse Assessment of Pesticide Compatibility

A suspension of *M. anisopliae* conidia was prepared by flooding 10-day-old cultures of strain 275-86 with 0.05% Triton X-100. The spores were removed by agitation with a metal rod and the conidial suspension was filtered through four layers of sterile coarse-mesh cheesecloth.

The suspension was centrifuged (10 min, 3000 rev min^{-1}) and resuspended in fresh 0.05% Triton X-100. The spore concentration was determined (as above) and adjusted to 10^7 conidia ml^{-1} by diluting with 0.05% Triton X-100.

Four hundred *Impatiens* (cv. Super Elfin Blush) were grown in peat compost in 0.4 l polythene pots. The plants were maintained on capillary matting in a shaded glasshouse with a minimum temperature of 15°C. Two hundred plants were drenched with the 10^7 conidia per ml suspension at a rate of 20 ml per pot, while the remaining plants received a similar volume drench of 0.05% Triton X-100. The plants were then laid out in a randomized block design on the glasshouse benches with 20 fungal and 20 Triton pots in each block (each pot was treated as a single plot for statistical analysis).

Vine weevil eggs used during this experiment were produced from cultures of adult weevils maintained on strawberry leaves at 20°C−22°C with a 16:8 hour light:dark regime. Eggs were collected after a 7 day laying period and incubated on moist filter paper for a further 3 days at 20°C. Ten 'tanned' eggs were placed on the roughened compost surface of each pot, 24 h after the fungal and control treatments. Five batches of 100 eggs were also placed on moist filter paper in Petri dishes and maintained under the same conditions as the pots so that egg hatch could be monitored.

All the fungicides (except quinomethionate and triforine) and insecticides (except aldrin and hostathion) in Table 1 were examined in the glasshouse experiment. The pesticide treatments were applied to the pots at approximately 16% egg hatch (7 days after egg application). Each pesticide was applied (20 ml per pot) at the recommended rate to 10 pots previously treated with the conidial suspension and to 10 untreated pots (one fungal and one Triton pot from each block). Each pot that had not been treated with one of the 16 pesticides received a 20 ml drench of 0.05% Triton X-100. Two sets of control pots were included in this design; 40 pots received neither the fungal nor the pesticide treatments and 40 pots were drenched with the *M. anisopliae* alone. The plants were maintained under glasshouse conditions for approximately 10 weeks. The plant tops were then removed at compost level and each pot was weighed and the numbers of live, dead and infected larvae per pot were determined by destructive assessment. The numbers of live larvae per pot were subjected to a square root transformation and the results were assessed by ANOVA.

A uniform 1 g compost sample was removed from each pot (with the exception of those in block I) and placed in a universal bottle containing 10 ml of 0.05% Triton X-100. The suspensions were then vortexed for 1 min and sonicated for 10 s prior to dilution. Two 0.02 ml aliquots of each suspension were plated onto a selective medium for *M. anisopliae* (SDA + 100 ppm dodine (Cyanamid UK, Gosport) plus 50 mg per l chloramphenicol plus 50 mg per l streptomycin sulphate), which had been developed by Gillespie & Casebow (unpublished) from the original work of Beilharz *et al.* (1982). The two replicate plates were incubated for 5 to 7 days at 23°C and the number of colony forming units (CFU) per plate was determined. The numbers of CFU per pot (CFU g^{-1} × pot weight[g]) were then assessed by ANOVA using a log transformation.

RESULTS

In vitro Tests on Germination and Growth

Two fungicides, chlorothalonil and zineb, totally inhibited germination of *M. anisopliae* spores, whereas the other fungicides and insecticides had no significant effect on germination (Table 2).

The mean radial extension rate of *M. anisopliae* was significantly reduced by the recommended rate of all the pesticides examined except propamocarb (Figure 1). Two fungicides, benomyl and carbendazim, totally inhibited growth at all three rates. In addition, growth was also prevented by 10 times the recommended rate of the fungicides, etridiazole, triforine and zineb, and the insecticides, dichlorvos and hostathion. In general, the reduction in mean radical extension rate of *M. anisopliae* was dependent on pesticide concentration.

TABLE 2. Effect of pesticides on the germination of *M. anisopliae* (275-86)

Pesticide	Germination (%)	Pesticide	Germination (%)
SDA alone	98.0		
Benomyl	97.3	Iprodione	100.0
Bupirimate	97.0	Propamocarb	97.3
Carbendazim	97.7	Pyrazaphos	99.0
Chlorothalonil	00.0	Quinomethionate	98.3
Etridiazole	97.7	Tolclofos-methyl	100.0
Fenarimol	96.3	Triforine	96.3
Furalaxyl	98.7	Zineb	00.0
Aldrin	98.7	Dichlorvos	98.0
Cypermethrin	99.0	Hostathion	99.7
Diazinon	99.3	Pirimicarb	99.7

Increasing the concentration of the chemicals (with the exception of fenarimol) from 0.1 to 10 times the recommended rate caused a significant reduction in fungal growth rate.

Glasshouse Assessment of Pesticide Compatibility

The mean weevil population was reduced ($P < 0.001$) by 88% on pots treated only with *M. anisopliae* (Table 3). This level of control was not significantly reduced by the subsequent application of pesticides at the recommended rate. The mean larval population on pots that received both pesticide and *M. anisopliae* drenches ranged from 0.1 to 1.0 (98%−82% reduction). Three of the chemicals examined, chlorothalonil, iprodione and zineb, did not have an additive effect with *M.anisopliae*, whereas the other 13 pesticides increased larval control by up to 11%. A log-link analysis of the data indicated that there were no significant interactions between the pesticides and *M. anisopliae*.

Some of the pesticides examined demonstrated insecticidal effects when applied without *M. anisopliae*. Diazinon, cypermethrin and dichlorvos reduced larval numbers by at least 75% ($P < 0.001$), whereas pirimicarb had no significant effect on the larval population. The high levels of larval control recorded with diazinon and cypermethrin meant that it was not possible to quantify any interaction between these products and *M. anisopliae*. Interestingly, the fungicides pyrazaphos and bupirimate significantly reduced larval numbers by 50% ($P < 0.001$) and 34% ($P < 0.05$), respectively.

The mean number of colonies recorded on the pots treated with strain 275-86 alone was 5.27×10^7 CFU per pot (Table 4). This represents a maximum recovery rate of 26.4% of the applied conidia, although conidia emanating from sporulating larvae may have boosted this figure. Two of the fungicides examined, tolclofos-methyl and zineb, significantly reduced the number of CFU per pot by 70% and 56%, respectively, compared with the pots treated with *M. anisopliae* alone ($P < 0.05$).

DISCUSSION

In this study, growth of *M. anisopliae* was more sensitive to pesticides than germination. The radial extension rate on SDA containing the recommended rate of all pesticides (except propamocarb) was reduced, whereas only chlorothalonil and zineb reduced germination. Zimmermann (1975) also concluded that germination of entomopathogenic Deuteromycetes was less sensitive to pesticides than growth. In contrast, Hall (1981) found that germination of *V. lecanii* was more sensitive. Germination and germ tube elongation, prior to penetration, is one of the most critical phases of the infection process and inhibition at this stage

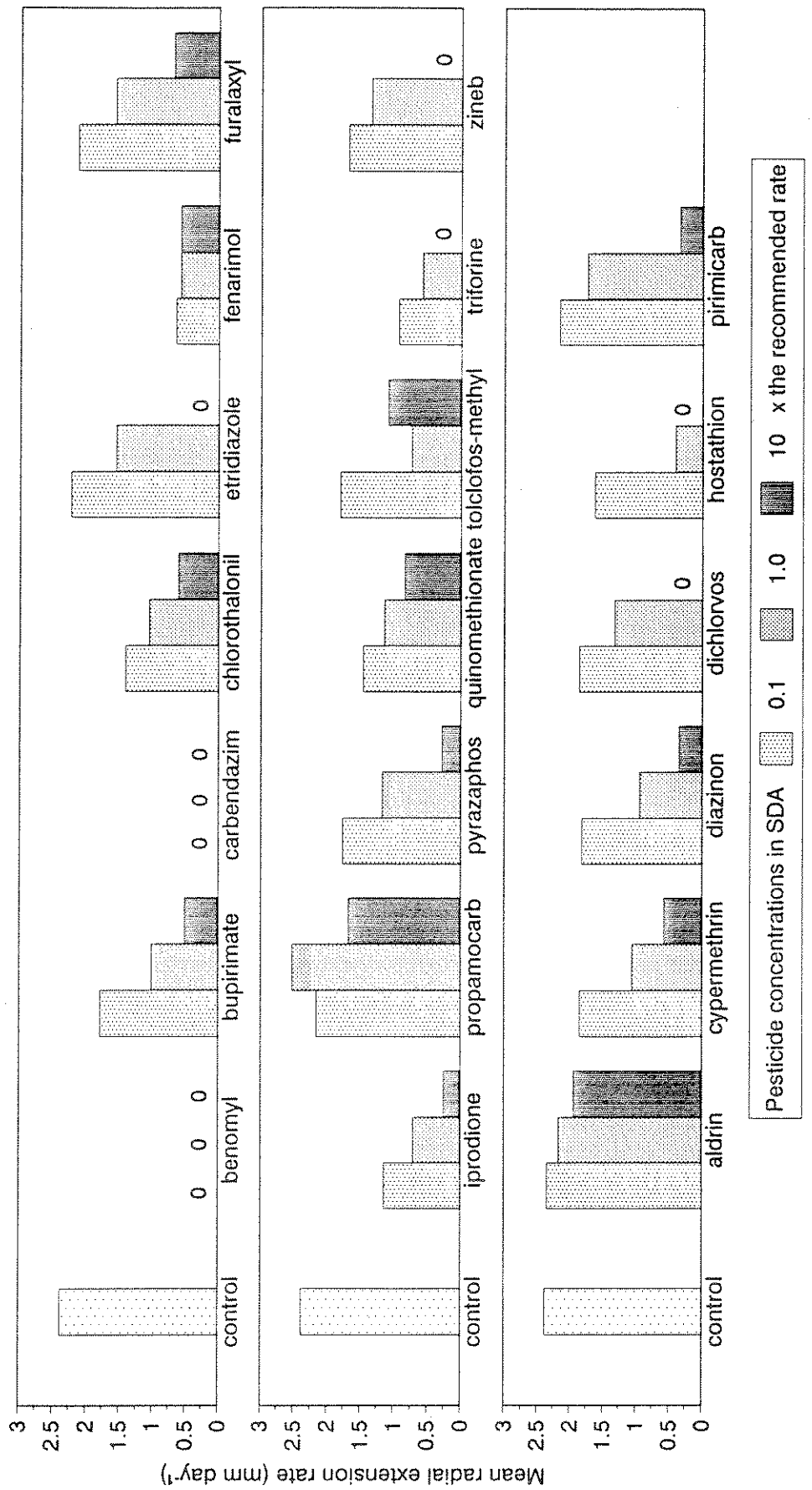

FIGURE 1. The mean radial extension rates of *M. anisopliae* (Strain 275-86) on SDA containing different pesticide concentrations (values are the mean of four replicate plates, SED <0.1 with 11 d.f.).

TABLE 3. Control of *O. sulcatus* larvae on *Impatiens* pots treated with combinations of *M. anisopliae* (strain 275-86) and pesticides

Pesticide	Alone			Plus *M. anisopliae*		
	Larvae per pot	(Square root trans.[a])	% Control[b]	Larvae per pot	(Square root trans.[a])	% Control[b]
Control	5.6	(2.30)	0	0.7	(0.58)	88
Benomyl	5.3	(2.26)	5	0.3	(0.24)	95
Bupirimate	3.7	(1.74)	34	0.5	(0.37)	91
Carbendazim	5.2	(2.21)	7	0.3	(0.30)	95
Chlorothalonil	6.2	(2.46)	−11	1.0	(0.82)	82
Etridiazole	4.1	(1.84)	27	0.3	(0.24)	95
Fenarimol	4.7	(2.13)	16	0.3	(0.30)	95
Furalaxyl	4.9	(2.18)	13	0.7	(0.35)	88
Iprodione	4.4	(2.03)	21	0.9	(0.66)	84
Propamocarb	4.5	(1.95)	20	0.2	(0.20)	96
Pyrazaphos	2.8	(1.44)	50	0.5	(0.50)	91
Tolclofos-methyl	3.7	(1.90)	34	0.7	(0.52)	88
Zineb	5.3	(2.22)	5	1.0	(0.69)	82
Cypermethrin	0.4	(0.34)	93	0.3	(0.30)	95
Diazinon	0.0	(0.00)	100	0.1	(0.10)	98
Dichlorvos	1.4	(1.04)	75	0.1	(0.10)	98
Pirimicarb	5.1	(2.21)	9	0.4	(0.27)	93

[a]Mean square root transformation of number of larvae per pot.
SEDs (with 356 d.f.) = 0.134 (control vs control); = 0.212 (control vs pesticide); = 0.268 (pesticide vs pesticide).
[b]Reduction in larval numbers compared with the control alone population.

will greatly reduce infection. This was illustrated by Wilding & Brobyn (1980) who reported reduced infection of aphids inoculated with *E. aphidis* four hours before the application of fungicide treatments, whereas the same treatments had little effect when applied after 22 or 70 h.

Pesticide compatibility has been extensively studied by incorporation of chemicals into agar, although some of the results are conflicting. Hall (1981) reported total inhibition of *V. lecanii* germination with mancozeb and benomyl, whereas Khalil *et al.* (1985) found that germination of the same species was only partially inhibited by mancozeb and not affected by benomyl. However, care should be exercised when comparing data in this way because of the differences in methods and dose rates. In addition, intra-specific variation in pesticide sensitivity of *V. lecanii* and *M. anisopliae* has also been observed (Olmert & Kenneth, 1974; Moorhouse & Gillespie, unpublished observation). Pesticide compatibility can also be examined by direct application of the compound to the agar surface after inoculation with the fungus. This can either be achieved using the treated filter paper technique described by Wilding (1972) or by spray application (Vänninen & Hokkanen, 1988). These two methods are more analogous to the application of entomogenous fungi in an insect pest management system and consequently the results are likely to be more meaningful.

The differences between the results of the glasshouse and laboratory pesticide compatibility experiments clearly demonstrate the limitations of *in vitro* assessment techniques. There was an indication that inhibition of germination might be linked to reduced infection rates because the two products which totally inhibited germination (chlorothalonil and zineb) had the lowest levels of larval control when combined with *M. anisopliae* in the pot experiment, although the reduction was not significant. The addition of pesticides to media represents a severe test for the fungus which it would not normally face in commercial practice. Conidia under glasshouse conditions would be exposed to an environment containing a variable concentra-

TABLE 4. Effect of pesticides on the number of CFU of *M. anisopliae* recovered from peat compost

Pesticide	$\log_{10}$ CFU per pot	Pesticide	$\log_{10}$ CFU per pot
M. anisopliae alone	7.722		
Benomyl	7.711	Furalaxyl	7.687
Bupirimate	7.470	Iprodione	7.815
Carbendazim	7.648	Pyrazaphos	7.537
Chlorothalonil	7.555	Propamocarb	7.742
Etridiazole	7.676	Tolclofos-methyl	7.197
Fenarimol	7.640	Zineb	7.366
Cypermethrin	7.602	Dichlorvos	7.637
Diazinon	7.676	Pirimicarb	7.732

SED = 0.1567 (126 d.f.).

tion of pesticide (due to uneven distribution) and factors such as rapid chemical breakdown and peat adsorption of the pesticide may become important. Wilding (1972) reported similar inconsistencies between *in vitro* and *in vivo* experiments examining the compatibility of *V. lecanii* (described as *Cephalosporium aphidicola*) with the systemic fungicide, triarimol. Fungal growth on nutrient agar was inhibited around filter paper discs treated with triariomol, whereas aphid infection was not reduced on cucumber plants previously drenched with the same product. Hall (1981) found that fenarimol was very toxic to *V. lecanii* when incorporated into agar; however it had little effect on aphid infection, except when applied simultaneously with conidia. In contrast, other pesticides inhibited germination and growth on agar and also reduced infection rates. Good correlation between pesticide suppression of germination and infection was also reported by Wilding & Brobyn (1980).

A number of products examined by Hall (1981) reduced infection rates when applied simultaneously with *V. lecanii* conidia, but inhibition was reduced when applications were separated by 24 h. Anderson & Roberts (1983) also concluded that fungal inhibition by pesticides could be reduced by separating the applications although, logistically, this might not be a satisfactory solution for a commercial grower. In addition, these workers considered that inhibition was frequently caused by formulation ingredients, particularly xylene-based solvents, rather than the active ingredient. It might therefore be possible to reduce the fungicidal and fungistatic properties of some of the more inhibitory products by using different formulations.

Öncüer & Latteur (1979) found that binapacryl was toxic to *E. obscura* conidia for at least 20 days in soil. Conidial infectivity was reduced by over 90% immediately after the application of four other fungicides, declining to less than 55% after 20 days. Conidial infectivity was not reduced when conidia were applied to the soil immediately after the application of copper oxychloride, dodine, benomyl or carbendazim. Simultaneous application of these fungicides with *E. obscura* conidia might not be advisable however, because Wilding & Brobyn (1980) reported that the germination of *E. aphidis* was totally inhibited by benomyl.

Prolonged conidial survival is likely to be an important characteristic of a successful microbial control agent for *O. sulcatus* and any reduction in survival would reduce the effective control period. Application of tolclofos-methyl and zineb to peat compost containing *M. anisopliae* conidia reduced the number of CFU per pot after 10 weeks by more than 56%. This reduction could have resulted from either mortality of the original conidia or inhibition of sporulation on infected larvae. Soil treatment with the herbicide, alachlor, reduced the number of *B. bassiana* CFU by 51% and halved the infection rate (Gardner & Storey, 1985). Reduction in sporulation has been frequently noted in *in vitro* experiments with pesticides (Gillespie, 1984; Vänninen & Hokkanen, 1988). Wilding & Brobyn (1980) reported that

conidial discharge from aphid cadavers as reduced by benomyl and tridemorph, but increased by captafol and captan.

The results of this experiment demonstrated that the control of *O. sulcatus* was not significantly increased by combining *M. anisopliae* with any of the pesticides examined. This contrasts with work on other entomogenous species where synergistic and/or additive interactions have been observed (Anderson *et al.*, 1989). These interactions are exploited for the control of *Leptinotarsa decemlineata* in Russia where applications of Boverin (*B. bassiana*) are combined with a low dose of an insecticide such as trichlorphon (Lipa, 1985). The sublethal insecticide dose weakens the insects and makes them more susceptible to infection. It is possible that the high levels of weevil control observed in the present experiment masked any interactions with the pesticides and further investigation might be justified using reduced doses of both the pesticides and *M. anisopliae*.

M. anisopliae clearly has a good deal of potential as a microbial control agent for *O. sulcatus* and the results from this study suggest that it will be possible to use *M. anisopliae* on protected crops as part of an integrated crop protection programme.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the funding for this project from the University of Bath, the Horticultural Development Council and the Agriculture and Food Research Council. We would also like to thank John Fenlon for statistical advice and Phil White for his helpful comments on the manuscript.

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