BIOLOGICAL METHODS TO CONTROL GREY MOULD OF STRAWBERRY

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ABSTRACT

A leaf-disc assay was developed to evaluate microorganisms for biological control of grey mould of strawberry, caused by *Botrytis cinerea*. Biocontrol efficacy of a total of 230 isolates of mycelial fungi, yeasts and bacteria from strawberry ranged from 0-100%. Organisms of various levels of effectiveness in the leaf-disc test were evaluated for fruit rot suppression in four field trials. Inocula were applied at weekly intervals from the green blossom bud stage to the white-pink fruit stage. Field observations correlated significantly with those on the leaf discs (r_s=0.45-0.97, P<0.1-0.01). Isolates of *Trichoderma viride* and *Gliocladium roseum* were highly suppressive to *B. cinerea* in all tests, and were as effective or more effective than standard captan sprays. Several fungi also suppressed sporulation of *B. cinerea* when applied to strawberry leaves colonized by the pathogen. A modified leaf-disc assay was used to screen 22 strawberry varieties for leaf resistance to *B. cinerea*; marked differences in sporulation density of the pathogen were observed among the varieties. Implications of the various observations for managing grey mould are discussed.

INTRODUCTION

Conventional fungicide programs used to control grey mould fruit rot of strawberry, caused by *Botrytis cinerea*, have become increasingly unacceptable to the grower and the public. Fungicide effectiveness is often inadequate, a circumstance variously attributable to difficulties in maintaining fungicide coverage on rapidly developing flowers of strawberry, and to development of fungicide tolerance to the pathogen (Northover and Matteoni 1986, Pepin and MacPherson 1982). Public concern with fungicide residues on the foliage and fruit has increased markedly. Alternative methods are needed for managing grey mould that are more effective than the available fungicides, and which avoid fungicide residues or other potential or perceived hazards in the fruit.

Microorganisms used as biocontrol agents represent a potential alternative to fungicides. Bhatt and Vaughan (1962) found that *Cladosporium herbarum* and *Pullularia* (=*Aureobasidium*) *pullulans* suppressed grey mould of strawberry fruits in a greenhouse study, but that *C. herbarum* was ineffective in the field. Tronsmo and Dennis (1977), however, observed that various *Trichoderma spp.* suppressed strawberry fruit rot in the field, and that the best isolate was as effective as dichlofluanid. Natural suppression of *B. cinerea* was

implicated in recent studies of the pathogen in strawberry leaves (Braun and Sutton 1988), and in field plots (McLean 1988).

Previous research has provided epidemiological and biological information for developing strategies to suppress grey mould in strawberry using microorganisms (Jarvis 1962b, Bristow *et al* 1986, Braun and Sutton 1987), but efficient screening techniques that allow identification of microorganisms of outstanding effectiveness are lacking. Dead strawberry leaves are the principal inoculum source of *B. cinerea* in Ontario strawberry fields (Braun and Sutton 1987). The fungus may infect the leaves, especially at the bud stage, and remain quiescent in epidermal cells until the leaves senesce and die (Braun and Sutton 1988). Dispersed conidia of *B. cinerea* infect the flowers from which mycelium invades the fruits (Jarvis 1962a, Bristow *et al* 1986). In the present study, techniques were developed for screening candidate organisms for biocontrol in the laboratory and in the field, and for screening strawberry genotypes for leaf resistance to *B. cinerea*. Effectiveness of selected biocontrol candidates was compared with that of captan for fruit rot management in field trials.

MATERIALS AND METHODS

Isolation and inoculum preparation of biocontrol candidates and *B.cinerea*

Mycelial fungi, yeasts and bacteria for evaluation in biocontrol tests were isolated from above-ground parts of strawberry plants in the field. Inocula of 230 biocontrol candidates for tests in the laboratory were produced on potato dextrose agar (mycelial fungi or yeasts) or nutrient agar (bacteria). For field tests, yeasts and bacteria were produced in a liquid medium (10 g sucrose, 10 g proteose peptone and 1 g yeast extract in 1 litre water), and mycelial fungi were produced on potato dextrose agar. Propagules of all organisms were centrifuged and resuspended in water containing surfactant (0.04 ml Triton XR/100 ml water). A haemacytometer was used to estimate inoculum densities of yeasts and mycelial fungi, and bacteria were estimated from standard curves of cfu/ml and absorbance at 550 nm. Inoculum density used in the controlled environment was 10^7 propagules/ml; in the field 10^6 spores of mycelial fungi/ml, 10^7 yeast cells /ml and 10^8 bacteria/ml were used. Spores of *B. cinerea* were produced on strawberry agar (Braun and Sutton 1987), recovered by aspiration into sterilized-distilled water plus surfactant, and diluted to 10^6 spores/ml and 2×10^3 spores/ml, respectively, for use in the controlled environment and in the field.

Inoculation

Strawberry leaf discs (1 cm in diameter) were inoculated with biocontrol candidates and *B. cinerea* in the laboratory by means of an aerosol sprayer. Strawberry plants in the field were inoculated using a compressed air sprayer of 7.6 litre capacity.

Estimation of grey mould

Infection and colonization of leaves and by *B. cinerea* in biocontrol studies were quantified indirectly by estimating sporulation density of the pathogen after the tissues were incubated on paraquat-chloramphenicol agar (PCA,

containing 20 mg paraquat, 200 mg chloramphenicol, and 10 g agar in 1 litre distilled water) modified from Bannon (1978). Sporulation was assessed as density and incidence of conidiophores on leaf discs and fruits. A 0-7 scale was used for estimating conidiophores on leaf discs, with classes equivalent to 0, 1-12, 13-24, 25-48, 49-100, 101-200, 201-300 and 301-400 conidiophores/disc, respectively. For estimating incidence of fruit rot in field plots, 100-150 fruits were harvested from each plot and incubated in separate compartments of plant flats at 20°C for 7 days, then examined for fruit rot and *B.cinerea*. Three to six replicate plots, each consisting of 4 m of matted row, were used for each treatment in the four separate field tests.

Biocontrol tests

Strawberry plants were grown in pots in the greenhouse and in field plots. A leaf disc assay was developed for rapid screening of biocontrol candidates in the laboratory. Discs were cut from the pot-grown plants and placed on a fibreglass screen which overlaid 3 layers of moist paper towel in petri plates. The discs were inoculated with candidate organisms, challenge-inoculated with *B. cinerea* after 24 h at 20-22°C, and transferred to PCA after another 24 h. Field tests were done at the Arkell and Cambridge Research Stations, near Guelph, Ontario, in 1989 and 1990. Fourteen organisms of various levels of effectiveness in the laboratory tests were evaluated in 3 field trials in 1989 and five were evaluated in 1990. Inocula were applied at weekly intervals when the strawberries developed from the green blossom-bud stage to the white-pink fruit stage. Captan (6.75 kg/ ha) was applied at the same time as a chemical check. Spores of *B. cinerea* were added to all inocula and to captan at a final concentration of 2×10^3 conidia/ml at the second week of treatment only.

The effectiveness of six organisms in suppressing sporulation of *B. cinerea* on leaves colonized by the pathogen was examined. In greenhouse studies, 10-day old strawberry leaves were inoculated with *B. cinerea*. The inoculated plants were kept in a mist chamber for 24 h and in the greenhouse for another 2 weeks, and were then inoculated with the biocontrol agents. After 3 days, discs were cut from the leaves, incubated on PCA and later assessed for sporulation of the pathogen. In the field, leaflets of overwintered leaves were inoculated with the biocontrol organisms on 26 May and 3 June (Trial 1). In a second study young leaves were inoculated with *B. cinerea* (10^5 conidia/ ml) on 13 May, and the leaves were treated with biocontrol agents on 13 and 20 June. Water and chlorothalonil 50 F (3.5 litres product /ha) were used as controls. Discs were cut from the leaves 3 days after the last treatment in each study and assessed for incidence of sporulation by the PCA technique.

Leaf resistance of strawberry varieties to *B.cinerea*

Leaf discs of 22 strawberry varieties were inoculated with *B. cinerea* (10^5 conidia/ml), and incubated for 24 h, surface sterilized in 70% ethanol and 0.6% NaOCl, and placed on PCA. Sporulation density of the pathogen was assessed as before. The experiment was repeated once.

Data analysis

Statistical computations were performed using Statistical Analysis System software (SAS Institute Inc., Cary, NC., USA). Observations of repeated

experiments were tested for homogeneity of variance and pooled accordingly. Cluster analysis ($P < 0.05$) was used to separate means (Scott and Knott, 1974).

RESULTS

Biocontrol studies in the laboratory

The biocontrol candidates variously suppressed *B. cinerea* on leaf discs by 0-100%. Five clusters (Scott and Knott 1974) of organisms were identified based on sporulation density of *B. cinerea* on the discs (Table 1). Isolates of bacteria and yeasts, and about 37% of the mycelial fungi were ineffective or only slightly effective in suppressing the pathogen (clusters a and b), however the efficacy of 27 % of isolates of mycelial fungi was moderately-high to high (clusters d and e). Cluster e included isolates of *Trichoderma viride, Gliocladium roseum* and *Myrothecium verrucaria*. Densities of conidiophores of *B. cinerea* on leaf discs treated with 5 organisms selected for field studies, and with water, are given in Table 2.

TABLE 1. Cluster analysis of the effects of biocontrol candidates on sporulation density of *Botrytis cinerea* on strawberry leaf discs in the laboratory.

Cluster	Mean number of conidiophores of *B. cinerea*/1-cm disc	Number of isolates of biocontrol candidate		
		Bacteria	Yeasts	Mycelial fungi
a *	260-187	6	4	20
b	184-106	16	17	50
c	102- 43	0	1	66
d	34- 14	0	0	28
e	10- 0	0	0	22

* Means in same cluster are not significantly different (cluster analysis, $P<0.05$).

Biological suppression of fruit rot in the field

Isolates of microorganisms that suppressed *B. cinerea* in the leaf disc assay were also relatively effective in suppressing grey mould fruit rot in the field (Table 2). Conversely, the white yeast, which was ineffective on leaf discs, was also ineffective in the field. Spearman's ranking values (r_s) in comparisons of observations in the laboratory and various field tests ranged from 0.45-0.97 ($P<0.1$ to 0.01). *Gliocladium roseum* suppressed fruit rot by 39-71% in the various field tests, and was as effective or significantly more effective than captan.

Biological suppression of sporulation by *B. cinerea* on strawberry leaves

Trichoderma viride, Penicillium sp., and *G. roseum* suppressed density of

sporulation by *B. cinerea* as effectively as chlorothalonil on leaves of strawberry in the greenhouse and in the field (Table 3). Isolates of *Fusarium sp*, and *M. verrucaria* were suppressive in the greenhouse but not in the field. The pink yeast was ineffective in all studies.

TABLE 2. Effects of various biocontrol candidates on estimated sporulation density of *B.cinerea* on leaf discs in the laboratory, and of the candidates and captan on estimated incidence of grey mould fruit rot in field studies in 1989 and 1990.

Treatment	Mean number of conidiophores /leaf disc (in laboratory)	Incidence of fruit rot (%) in the field 1989 Trial 1	1989 Trial 2	1989 Trial 3	1990
Water (control)	260 a *	59 a	71 a	58 a	44 a
White yeast	182 a	43 b	72 a	53 a	43 a
Trichothecium roseum	97 b	27 c	41 c	36 b	36 b
Epicoccum nigrum	29 c	43 b	51 c	25 c	35 b
Trichoderma viride	3 d	38 b	37 c	28 c	35 b
Gliocladium roseum	0 d	25 c	43 c	17 c	20 c
Captan (control)	---	40 b	56 b	23 c	20 c

* Means in a column followed by same letter are not significantly different (cluster analysis, $P<0.05$).

TABLE 3. Effects of biocontrol candidates and of chlorothalonil on sporulation of *B. cinerea* on strawberry leaves in the greenhouse and in the field.

Treatment	Sporulation density on leaves in greenhouse (conidiophores / leaf disc)	Incidence of sporulation on discs of leaves in the field (%) Trial 1	Trial 2
Water (control)	120 a *	25.7 a	18.7 a
Pink yeast	112 a	27.0 a	16.4 a
Fusarium sp.	28 b	23.0 a	23.6 a
Myrothecium verrucaria	2 c	22.0 a	14.0 a
Trichoderma viride	4 c	13.3 b	2.8 b
Penicillium sp.	3 c	9.3 b	0.0 b
Gliocladium roseum	0 c	10.7 b	0.0 b
Chlorothalonil (control)	3 c	9.0 b	3.5 b

* Means (of 6 replications) in a column followed by same letter are not significantly different (cluster analysis, $P<0.05$)

Leaf resistance of strawberry varieties

Sporulation density of *B. cinerea* was significantly lower on the leaf discs of 10 of the 22 strawberry varieties tested (Table 4). Sporulation density in 9 of the 10 varieties that were relatively resistant was roughly 50 % lower than in the 12 more susceptible varieties. Sporulation density in Midway, however, was 84-90% less than in the 12 susceptible varieties.

TABLE 4. Sporulation density of *B. cinerea* on leaf discs of different strawberry varieties.

Variety	No.of conidiophores of *B.cinerea* / 1-cm leaf disc	Variety	No.of conidiophores of *B.cinerea* /1-cm leaf disc
Chandler	146 a *	Sparkle	94 a
Selva	139 a	Pajaro	79 b
Gu62E55	123 a	V7261-3	59 b
MicMac	122 a	Vesper	56 b
Raritan	120 a	V7251-1	56 b
Vibrant	115 a	V7210-5	53 b
Redcoat	111 a	Guardian	49 b
Gov. Simcoe	106 a	Kent	48 b
Settler	106 a	Bounty	46 b
Glooscap	100 a	Vantage	39 b
Veestar	99 a	Midway	15 b

* Means (of 6 replications) followed by same letter are not significantly different (cluster analysis, $P<0.05$).

DISCUSSION

Isolates of *T. viride* and *G. roseum* were highly suppressive to *B. cinerea* in the leaf disc assays, and were generally as effective or more effective than standard captan sprays in suppressing grey mould fruit rot in the field. Other organisms of low or intermediate effectiveness against the pathogen in the laboratory also performed similarly in the field. The generally high r_s values obtained in comparisons of the laboratory observations and data of the four field trials indicated that the leaf disc assay was valid for preliminary screening of candidate organisms. Other isolates that were highly suppressive in the laboratory but were not tested in the field also may be of value in managing grey mould. Our observations that *T. viride* effectively suppressed *B. cinerea* support earlier results of Tronsmo and Dennis (1977). Unfortunately, *T. viride* may itself under some conditions contribute to fruit rot in strawberry. However, *G. roseum* was significantly more effective than *T. viride* in two field tests, and is not known to contribute to fruit rot. This fungus, and others that were highly effective in

the laboratory tests warrant further investigation as biocontrol candidates.

The observed suppression of sporulation of *B. cinerea* on the leaves colonized by the pathogen in the greenhouse and in the field by *T. viride*, *Penicillium sp.*, and *G. roseum* indicated that these, and perhaps other organisms may have value in reducing inoculum density of the pathogen in strawberry fields. Suppression of inoculum production by the pathogen is probably more feasible when the fungus persists as mycelium in strawberry leaves, as in Ontario (Braun and Sutton 1987), than when it produces an abundance of sclerotia, as in Scotland (Jarvis 1962b). Suppression of inoculum at the source by saprotrophic organisms or by fungicides (Braun and Sutton 1986) may reduce infection frequencies of flowers and fruits (Braun and Sutton 1988).

From the observations of the various strawberry varieties, leaf resistance is another method of suppressing inoculum production by *B. cinerea*. The leaf disc technique was an effective and efficient means for quantifying resistance of genotypes to the pathogen, and is suitable for rapid screening of varieties and breeding materials.

An important implication of these various observations is that grey mould fruit rot may be managed in strawberry crops in Ontario without the use of fungicides. Protection of the flowers and fruits against infection by the pathogen is a key approach in biocontrol strategies. However reduction of inoculum production on dead strawberry leaves by means of microorganisms applied to the leaves, or through resistance of the leaves to *B. cinerea*, may also contribute effectively to disease management.

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INTERACTION OF CONVENTIONAL INSECTICIDES WITH *Bacillus thuringiensis* AGAINST THE MEDITERRANEAN FRUIT FLY, *Ceratitis capitata* (Weid.)

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ABSTRACT

The bacterial insecticide *Bacillus thuringiensis* var. *israelensis*, serotype H-14 and the insecticides cypermethrin, dimethoate and methomyl were tested against larvae of the Mediterranean fruit fly, *Ceratitis capitata*. Combinations of LC10, LC30 and LC50 levels of the three insecticides with LC15 of *B. thuringiensis* showed potentiation of toxicity to the larval stage. The joint effect was also found to induce retardation and anomalies in the development of treated larvae.

INTRODUCTION

The Mediterranean fruit fly, *Ceratitis capitata*, is an insect pest of considerable economic importance throughout the Mediterranean region and where there are similar climates worldwide. Its host range includes 200 varieties of fruit crops (Christenson & Foote, 1960). Chemical insecticides are still the common means of control, despite their health and environmental hazards (Attala, *et al.*, 1981).

The present study was planned to explore the possibility of using *Bacillus thuringiensis* either alone or in mixtures with sub-lethal levels of some insecticides to control *C. capitata*.

MARTERIAL AND METHODS

Insecticides tested

Three formulated insecticides, dimethoate (40% EC), cypermethrin (20% EC) and methomyl (90% SP) were tested under standard laboratory conditions against the larval stage and the LC50 values were computed using regression probit analysis (Finney, 1952).

The bacterial insecticide tested

The strain of Bacillus thuringiensis var. israelensis, serotype H-14, was obtained from Canada Agriculture. It is a dry powder containing 27000 IU/mg. This strain was reported to be highly pathogenic to dipterous insects (Lacey & Undeen, 1986).

The insect culture

The culture of C. capitata used was obtained from the Ministry of Agriculture, Cairo. The culture was reared and maintained at 25 + 2 C, and 50-60% r.h. Adults were fed on sucrose with protein hydrolysate (Buminal) (Arruda, et al., 1982). The larvae were reared on an artificial diet (El-Abassi, 1988) composed of:

citric acid	8 g	molasses	200 g
sodium benzoate	8 g	wheat bran	250 g
Brewer's Yeast	180 g	water	1000 ml

Toxicity to larvae

The stomach toxicity to C. capitata larvae was assessed by mixing the larval diet (20 g in each petri dish) with 3 ml of a series of concentrations in distilled water of either B. thuringiensis or insecticide, followed by ten 2nd instar larvae in each of five replicates. Larval mortality was recorded after 48 h.

Joint effect of insecticides and B. thuringiensis on larvae and their development

The respective amount of LC15 for B. thuringiensis (2.0 mg/Kg) were combined with either LC10, LC30 or LC50 levels of each insecticide (Table 1) and mixed together in the larval diet. Larval mortality was recorded after 48 h and any surviving larvae were allowed to develop. The number of pupae and emerging adults and their malformations or retardation were also recorded.

RESULTS AND DISCUSSION

Toxicity to larvae

The data in Table 1 indicate that dimethoate was more toxic to larvae followed by methomyl and then cypermethrin. The known relative higher polarity of dimethoate and methomyl supports their superiority to cypermethrin regarding their stomach toxicity to C. capitata (El-Sebae, 1985). The acute toxicity of B. thuringiensis was less than that of the synthetic insecticides.
The results showed that insecticides incorporated in the agar medium, at levels lethal to C. capitata, did not reduce the number of replicated bacterial cells after 144 h, even up to 1

mg/Kg, which far exceeds the LC50 values. Similar data on compatibility were reported by Habib and Garcia (1981).

TABLE 1: Toxicity to *Ceratitis capitata* larvae.

Material	LC50 mg/Kg	Confidence limits	Slope	LC30 mg/Kg	LC10 mg/Kg
Dimethoate	0.042	0.014-0.083	1.46	0.021	0.0085
Methomyl	0.090	0.088-0.092	2.74	0.062	0.037
Cypermethrin	0.210	0.186-0.234	2.13	0.12	0.05
B. thuringiensis	4.0	3.98-4.02	3.26	-	-

Synergism of *B. thuringiensis* and insecticides at sub-lethal levels

Natural mortality did not exceed 4% in larvae and 2% in pupae and adults. Abbot's formula was used for correction of the toxicity data. Malformations were not observed in the controls. Data in Table 2 suggest synergism between *B. thuringiensis* and insecticides against the larval stage, especially with dimethoate. Some of the apparent synergism could have been due to variation between batches of *C. capitata*.

TABLE 2: Joint effects of *B. thuringiensis* and three insecticides on larval development of *C. capitata*

Combination	% larval mortality(48 H)	% dead pupae	Emerged adults % normal	% malformed
LC15(B) + LC10(D)	46.0	8.0	26.0	20.0
LC15(B) + LC30(D)	64.0	10.0	14.0	12.0
LC15(B) + LC50(D)	84.0	8.0	4.0	4.0
LC15(B) + LC10(M)	42.0	14.0	28.0	16.0
LC15(B) + LC30(M)	60.0	6.0	24.0	10.0
LC15(B) + LC50(M)	82.0	0.0	14.0	4.0
LC15(B) + LC10(C)	40.0	10.0	36.0	14.0
LC15(B) + LC30(C)	56.0	6.0	28.0	10.0
LC15(B) + LC50(C)	78.0	0.0	16.0	6.0

B = *B. thuringiensis* M = methomyl
D = dimethoate C = cypermethrin

These results support the suggestion of using combinations of bacterial and chemical insecticides for more efficient performance and less pollution of the environment. A similar conclusion was reported by Kennedy and Oatman (1976).

Long-term effects on development

The resluts in Table 2 emphasize that the larvae which survive the treatment are subject to retadation of adult emergence for 9-11 days, and do not always develop into normal adults. Several cases of malformation of retarded pupae and emerging adults were well demonstrated. Similarly, Briggs (1960) reported that Bacillus spp. caused a reduction of adult house fly emergence by a deleterious effect on the development of the immature stage. This long term effect of B. thuringiensis in mixtures with conventional insecticides paves the way for new advantageous uses in the context of integrated pest management and rationalization of the use of synthetic insecticides.

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DEVELOPMENT OF A NOVEL Bt STRAIN FOR THE CONTROL OF FORESTRY PESTS

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ABSTRACT

BIODART™ is a new water-based Bacillus thuringiensis-related bioinsecticide developed for the control of spruce budworm (Choristoneura fumiferana var. clemens), gypsy moth (Lymantria dispar) and forest tent caterpillar (Malacosoma disstria) in forestry. The product contains a novel and indigenous Canadian B.t. strain (A20) selected for its higher intrinsic potency and broad spectrum of activity when compared with HD-1 and NRD-12. Trials conducted in three Canadian provinces in the summer of 1988 and 1989 demonstrated the effectiveness of the product in controlling spruce budworm and gypsy moth larval populations and in significantly reducing defoliation. The A20 strain also shows good broad spectrum activity against a range of important agricultural pests and its potential for use in agricultural outlets is discussed.

INTRODUCTION

Bacillus thuringiensis variety kurstaki (mainly the strains HD-1 and NRD-12) has been extensively used in North America for the larval control of spruce budworm, gypsy moth and, more recently, forest tent caterpillar (Malacosoma disstria) in forestry (Dubois, 1981; Dulmage, 1971). The field efficacy of Bt products is, however, often limited by poor persistence attributed to solar radiation sensitivity, slow speed of action and narrow spectrum of activity.

A screening programme was undertaken by ICI to identify natural isolates of Bt which are intrinsically more active than the standard commercially available strains against a range of key forestry and agricultural pests and show improved resistance to environmental stresses. A novel B.thuringiensis var. kurstaki strain named A20 was isolated at the ICI Biological Products laboratories in Mississauga, Canada, after screening 500 naturally-occurring B.thuringiensis isolates against C.fumiferana and L.dispar and various agricultural pests including Trichoplusia ni, Heliothis zea and Spodoptera exigua. A20, a Canadian indigenous strain, was selected on the basis of its intrinsic potency and broad spectrum of activity against these pests. The strain was further characterized and compared to other Bt strains including HD-1.

A water-based formulated concentrate at 64 BIU/US gallon (16.9 BIU/L) was developed and field trials were conducted in three Canadian provinces (Québec, Ontario and New Brunswick) in the summer of 1988 and 1989. Concurrently a full toxicology package including acute toxicology studies and environmental toxicology (birds, mammals and non-target invertebrates) on both the active ingredient and the end use product was prepared for Canadian registration purposes. The product was registered at the beginning of 1990 and was launched a few months later by ICI Forest Products. The A20 strain also shows good broad spectrum activity against a range of important agricultural pests. Reported here are some of the laboratory studies and field trial data related to the product development.

MATERIALS AND METHODS

Bt strain isolation and characterization

More than 500 Bt strains were recovered from soil, grain dust samples and homogenized dead insects.

Biomass consisting of a mixture of vegetative and sporulated cells and free spores and crystals was produced for each single strain by solid-state fermentation using a cotton seed flour-supplemented medium and diet-bioassayed against Trichoplusia ni, Choristoneura fumiferana, Lymantria dispar, Heliothis virescens, Heliothis zea, Spodoptera exigua, Spodoptera frugiperda and Ostrinia nubilalis using a methodology similar to the one described by Dulmage et al. (1971).

A20 biochemical markers were determined using API-ZYME™ and ID-IDENT™ kits (API Laboratory Products, Canada). Fatty acid profiles were determined by gas chromatography (see Minnikin and Goodfellow (1981) for an extensive review). Flagellar serotyping was performed according to De Barjac and Bonnefoi (1962).

Forestry Field Trials

In each Canadian province where A20 was tested, suitable sectors (including both treated and control plots) were selected from province-wide surveys of the overwintering larval populations. For spruce budworm, plots chosen were composed of young stands 30 to 50 years old, each measuring between 36 and 100 hectares and easily accessible by land. They were also selected on relatively flat land, to facilitate aerial spraying operations. The majority of the trees sprayed were balsam fir (Abies balsamea), approximately 10 to 20 m high and spruce (Picea spp.) averaging 11 metres in height. Typically, sixty balsam fir trees in each treatment site were selected and numbered for sampling purposes (larval mortality and Bt deposit). Cessna AG Truch Spray and Piper Pawnee aircraft equipped with Micronaire AU 4000 or AU 5000 rotary atomizers were used to apply Bt at a rate of 30 BIU/ha (regardless of the product used) (one single application) with a nominal swath width of 33 metres. Bt was applied when larval development was between the 4th and 5th instar which is usually when the shoots are expanding (beginning of June).

Selection criteria for gypsy moth efficacy trials included the presence and frequency of visible egg masses with larvae successfully emerging in the laboratory and presence of preferred host species (oak [Quercus spp.] and maple [Acer spp.]) as dominant and/or codominant species. Bt was

applied at the rate of 30 BIU/ha in two applications, one week apart and assessed according to Talerico (1981). Treatment was started before insect population development reached the 5th instar (mid-May and onwards depending upon the geographical location of the plots).

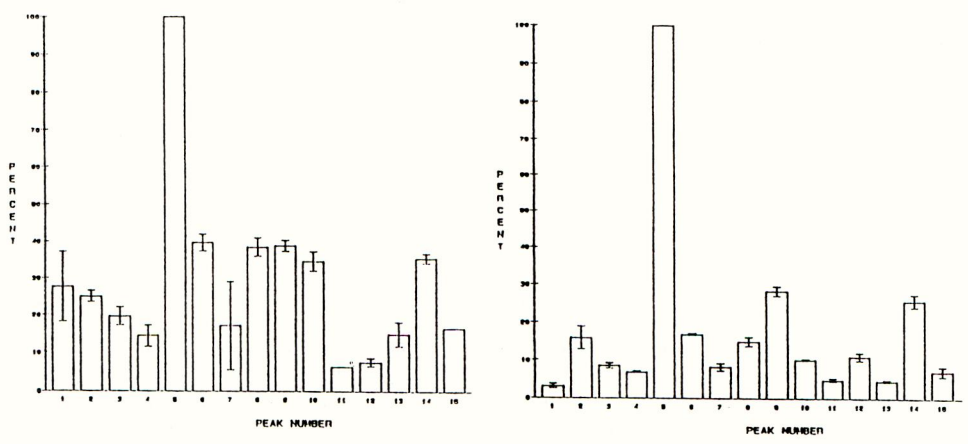
Selection criteria for forest tent caterpillar are the same as those described for gypsy moth except that the dominant host species are maple and aspen (Populus spp.). Bt application rate was 20 BIU/ha using 4 L/ha in a single application when insect population development reached the 3rd to 4th instar. The blocks were identified and located using aerial photos and insect surveys provided by the Canadian Forestry Service. All blocks were sprayed at the same time. Larval development was monitored throughout the field trial program from the time the larvae began to emerge in Mid-May until pupation in July. Each week and occasionally more often, branches from tree crowns were randomly pruned and the first 100 larvae were examined to determine their stage of development and viability.

RESULTS

The screening of 500 new Bt isolates allowed ICI to isolate a novel Bacillus thuringiensis strain named A20 from a Canadian soil source. The strain was serotyped and was shown to have the same flagellar serotype as HD-1 referred to as 'kurstaki'. Even though the two strains share similar biochemical markers, we found differences in the following markers: β-glucosidase, leucine hydrolysis, proline hydrolysis, histidine hydrolysis, acid phosphatase 2, Voges-Proskauer, pyroglutamic acid arylamidase and phosphoamidase.

Fatty acid profiles of A20 and HD-1 were also determined and are compared in Figure 1.

FIGURE 1. Comparative fatty acid profiles for A20 and HD-1



A20 HD-1

Note: The areas obtained for the various methyl esters corresponding to the free fatty acids were normalized to the base peak (Me. 13-methyltetradecanoate) and averaged.

A20 was shown to have enhanced activity against various foresty and agricuture-related Lepidoptera. Comparative diet bioassay data are shown in Table 1.

TABLE 1. Relative potency of Bacillus thuringiensis A20 when compared to HD-1 and NRD-12 strains.

Insect	Strain	LC50 (ppm)	Relative Potency
Choristoneura fumiferana	A20	2.1	5.1
	HD-1	10.5	
Lymantria dispar	A20	1.7	2.5
	HD-1	4.2	
Trichoplusia ni	A20	0.9	6.3
	HD-1	5.7	
Plutella xylostella	A20	0.4	10.0
	HD-1	4.0	
Heliothis zea	A20	21.0	2.5
	HD-1	52.5	
	NRD-12	20.0	1.0
Heliothis virescens	A20	0.5	2.6
	HD-1	1.3	
	NRD-12	1.7	3.4
Spodoptera exigua	A20	21.0	1.7
	HD-1	35.0	
	NRD-12	17.0	0.8
Spodoptera frugiperda	A20	21.0	1.2
	HD-1	26.0	
	NRD-12	13.0	0.6
Ostrinia nubilalis	A20	1.2	1.7
	HD-1	2.0	
	NRD-12	4.0	3.3

To test A20 biological activity in the field against forestry pests, a water-based formulation was developed at a biological activity of 16.9 BIU/L (64 BIU/US gallon) and applied at a volume of 1.7 L/ha corresponding to a dosage of 30 BIU/ha. Table 2 summarizes the Bt deposit observed after aerial spraying against spruce budworm whereas biological effectiveness is described in Table 3. Data related to one of our gypsy moth field trials are listed in Table 4.

TABLE 2. Spray deposit comparison of A20 and HD-1 on balsam fir foliage in Quebec, 1989.

Treatment	Volume L/ha	Mean Spray Deposit Drops/Needle
A20	1.7	0.306 ± 0.165* (14)**
HD-1	1.7	0.282 ± 0.345 (28)

* Standard Deviation
** number of branches processed (from the top of the tree).

TABLE 3. Larval mortality and reduction in defoliation observed with A20 and HD-1 against spruce budworm in Quebec, 1989.

Population (Larvae/Branch)[1] Pre-Spray May 1989	Post-Spray June 1989	Corrected % Mortality[2] 2 Days	Final	% Reduction[3] in Defoliation
A20 Treatment				
15.5 [12.9; 18.6] N=58[4]	1.1 [1.0; 1.1] N=58	46.3 ±8.5 N=28	93.5 ±3.2 N=58	21.5 ±7.3 N=57
HD-1				
9.1 [8.0; 10.5] N=116	1.3 [1.2; 1.4] N=116	21.3 ±10.4 N=57	64.2 ±6.7 N=116	44.5 ±4.1 N=116
Control				
13.5 [11.2; 16.2] N=110	2.1 [1.8; 2.4] N=110	0.0 ±6.1 N=54	0.0 ±11.1 N=110	-18.8 ±6.1 N=104

1 Means retransformed from logarithmic means. The numbers in square brackets represent ca. 95%.
2 100 x (mortality in treatment - mortality in control)/(100 - mortality in control)
3 100 x (expected defoliation [according to a forecast model based on initial budworm density] minus observed defoliation)/expected defoliation.
4 Number of trees samples (one 45-cm branch/tree, taken from the top)

TABLE 4. Results of spray efficacy of A20 and HD-1 against gypsy moth in Ontario in 1989.

Treatment	Egg Masses/ha May 89	Egg Masses/ha Nov 89	% Population reduction*	Defoliation (%)
A20	37,000	7,600	81	50
Leading Commercial Product	4,100	7,300	Increase	40
Control	30,500	33,500	0	85

* Populations based on egg masses/ha.

Finally, results obtained in the biological control of forest tent caterpillar are contained in Table 5.

TABLE 5. Results of spray efficacy of A20 and HD-1 against forest tent caterpillar in Ontario in 1989.

Treatment	Population Pre-Treatment (larvae/tree)	Population Post-Treatment (pupae/tree)	Defoliation %
A20	7034	2.6	6.3
Commercial Product	5452	4.6	4.6
Control	7206	61	23.6

DISCUSSION

ICI's Bt product contains an indigenous, natural Bacillus thuringiensis var. kurstaki strain that has been shown to have, in general, a superior intrinsic activity and a broader spectrum of activity than the standard HD-1 and NRD-12 strains. Under laboratory conditions, ICI's Bt product has also demonstrated good activity against other forestry pests including western spruce budworm (Choristoneura occidentalis), eastern hemlock looper (Lambdina fiscellaria), jack pine budworm (Choristoneura pinus pinus) and white marked tussock moth (Orygia leucostigma) (data not shown). The product is also effective against a wide range of important agricultural pests and field trials are currently under way to evaluate its development potential in agricultural outlets.

Since A20 was isolated from an environment that could have been exposed to Bt spraying, an exhaustive microbiological characterization was conducted to confirm that it was a different strain from HD-1 even though they both had the same flagellar serotype.

Forestry field trials conducted in three different Canadian provinces in 1988 and 1989 against spruce budworm indicated that the efficacy of A20 was comparable to the different commercial HD-1-based products used throughout their Bt spraying programme. Data presented in Table 3 show higher larval mortality than HD-1 against spruce budworm. Prevention of defoliation, even though there was a significant reduction when compared to the control, was not as good as HD-1. However, due to meteorological conditions, A20 was applied 2 weeks later allowing the larval population (which was higher in block 3) to destroy a fair proportion of the foliage before treatment.

Results against both gypsy moth and forest tent caterpillar are also excellent in terms of reducing insect population levels. With respect to defoliation, ICI's product provided adequate foliage protection (50% defoliation or less being considered as adequate by the users). When compared to HD-1, results are more dramatic considering the higher pre-spray population levels encountered in ICI's plots.

For the main pests (spruce budworm and gypsy moth) encountered in Eastern Canada, Bt is applied as a neat solution of 12.7 or 16.9 BIU/L (48 or 64 BIU/US gallon) corresponding to application volume of 2.4 and 1.7 L/ha respectively for a final dosage of 30 BIU/ha. Size and density of droplets are determining factors with respect to population control and defoliation, and are dependent upon the product rheology. The deposit study after A20 application as summarized in Table 2 shows a number of droplets very close to the optimum level of 0.75 droplets per needle. In a similar field trial, ICI's product showed a deposited droplet spectrum in which 66% of the droplets were less than 50 µm (considered as very good by the users).

Furthermore, A20 water-based formulation was shown to be non-corrosive with the right viscosity and yield point to provide the customer with a product easy to spray and dispose of after use.

Finally, as part of ICI's commitment to the development of environmentally-safe insecticides, we investigated the environmental fate and impact of A20, both in the field and in the laboratory through microcosm studies. Previous studies on Bt in general and toxicology tests conducted on A20 had indicated that there should be no impact on aquatic or terrestrial animals and plants, but we wished to assess the possible effects of A20 on soil microflora. No significant changes in microbial populations were found following spraying of A20 even at 100 times the concentration used in aerial applications. Results from the environmental fate study showed no significant spore build-up in either the organic or mineral layers in the soil over a period of 11 months. Also there was no persistence in running water following aerial spraying.

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PASTEURIA PENETRANS AS A BIOCONTROL AGENT OF MELOIDOGYNE JAVANICA IN THE FIELD IN MALAWI AND IN MICROPLOTS IN PAKISTAN

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ABSTRACT

Pasteuria penetrans was applied to sterilized soil in microplots in Pakistan. Inoculations with *Meloidogyne javanica* led to less galling and fewer egg masses on tomato roots than in similar soil without *P. penetrans*; shoot growth was also greater. These differences were accentuated in a second cycle after incorporating the roots of the first crop in the soil. In field plots in Malawi *P. penetrans* was mixed with soil at two sites where there was an indigenous population. Where roots were re-incorporated into a second crop galling was decreased regardless of whether *P. penetrans* was artificially applied or not.

INTRODUCTION

Some promising results have been obtained in pot experiments with *Pasteuria penetrans*, a bacterial pathogen of root-knot nematodes (*Meloidogyne* spp.) (Sayre, 1980; Stirling, 1981; 1984; Dube & Smart, 1987; Channer & Gowen, 1988). Because of the difficulties of producing inoculum few evaluations have been extended to test its efficacy in the field.

MATERIALS AND METHODS

Field studies in Malawi

Similar experiments were conducted at two sites, Bvumbwe Agricultural Research Station and Kwenengwe Horticultural Estate, which are 4 km apart. The sites had previously been planted to tomatoes and were infested with *Meloidogyne javanica*. Prior to establishing the experiments it had been discovered that there was a natural infestation of *P. penetrans* (Pp) in the selected sites. Tomatoes (cv. Moneymaker) were planted in a completely randomised block design with six replicates and three treatments : untreated control, *P. penetrans* (a blend of isolates prepared at Reading University), and carbofuran (10% AI granules). Each plot consisted of three rows of 14 plants, with six experimental plants finally assessed from each central row.

The Pp was applied as a spot treatment; approximately 27 litres of soil were taken at each plant site and a preparation of Pp in powdered tomato roots (Stirling & Wachtel, 1980) was mixed thoroughly in the soil at a rate of 2000 spores/g. The treated soil was replaced and tomatoes were

planted five days later. The carbofuran (4 g per plant) was applied similarly.

The experiments were planted in October 1989 and sprinkler irrigated until the rainy season started. The fruit was harvested over a three week period and at its termination an assessment was made of root galling.

At the Kwenengwe site, roots from all the experimental plants in the first crop were dried, crushed and incorporated into the same planting stations, with the intention of increasing the Pp inoculum. Carbofuran was reapplied to plots of the third treatment and the experiment replanted. Fruit was harvested from this second crop cycle and, at termination, the levels of galling in both experimental and guard row plants were assessed.

Microplot studies in Pakistan

Microplots on the University Farm at Faisalabad were filled with a sandy loam soil that had previously been sterilized with Formalin. Tomatoes (cv. Moneymaker) were planted in plots which had earlier been treated with an isolate of P. penetrans (Pp 3) at a dose of 5000 spores/g to a depth of 7.5 cm, other plots received no P. penetrans. One week later plants in soil with and without Pp were inoculated with 2000 M. javanica juveniles, a third treatment received neither nematodes or bacteria.

The experiment was run for six weeks until the first generation of nematodes reached maturity and Pp infected females contained endospores. Plants were then lifted and shoot weights and number of root galls and egg masses were recorded. The roots were chopped and re-incorporated into the plots, tomatoes were re-planted after 15 days and nematodes re-inoculated.

RESULTS

At Bvumbwe, the addition of P. penetrans or carbofuran had no effect on the amount of root galling but at Kwenengwe Pasteuria and carbofuran had a significant effect on the root galling in the first cycle but Pasteuria appeared less effective in the second cycle (Table 1). Second cycle guard row plants at Kwenengwe were more heavily infested - these plants had not had Pp applied (because of short supply of material) and roots of the first crop cycle guard plants had not been re-incorporated.

TABLE 1. Mean galling indices (on a scale 0-10) per root system obtained per treatment at two sites in Malawi

	Bvumbwe	Kwenengwe		
		1st cycle	2nd cycle expt.	2nd cycle guard
Untreated Control	6.50	2.39	2.26	4.00
Pp. Blend	6.22	1.06	1.21	3.22*
Carbofuran	5.42	0.53	0.89	3.39
S.E. Diff	0.77	0.65	0.58	0.72

* Pp Blend not applied to guard plants

Yields of tomatoes did not show significant differences at either Malawi site (Table 2).

TABLE 2. Yield (Kg/plant) from tomatoes grown at two sites in Malawi

	Bvumbwe	Kwenengwe	
		1st Cycle	2nd Cycle
Untreated control	2.43	1.21	0.87
Pp. Blend	2.25	1.30	0.85
Carbofuran	2.65	1.28	1.13
S.E. Diff	0.37	0.19	0.14

In Pakistan the use of P. penetrans in microplot soil to which M. javanica was applied significantly improved the shoot growth of tomatoes in both crop cycles (Table 3). P. penetrans also decreased the amount of root galling and numbers of egg masses that were visible on the roots.

TABLE 3 Shoot weights, numbers of galls and M. javanica egg masses on two cycles of tomato plants grown for 6 weeks in field microplots at Faisalabad, Pakistan.

	Shoot wt (g)	galls	egg masses
Crop 1			
M. javanica	8.80	64	34
M. javanica + Pp.	9.75	48	21
Control	13.05	-	-
Crop 2			
M. javanica	11.02	133	88
M. javanica +Pp	14.99	25	8
Control	14.70	-	-

Means of 10 Replicates. Differences between treatments M. javanica/ M. javanica + Pp significant P=0.05 shoots; P=0.01 galls and egg masses.

DISCUSSION

The difficulty in obtaining sufficient Pp inoculum for use in the field is a major limitation to its widescale use. For subsistence and market gardeners it may be possible to increase the amount of Pp spores in soil by the repeated incorporation of root systems containing Pp infected nematodes. In this respect the results from the microplots in Pakistan were most promising whereas those from the field sites in Malawi were less so. However, it is notable that guard row plants in all treatments were significantly more infested than experimental plants. Root-incorporation thus had a suppressive effect on nematode reproduction, regardless of whether Pp had originally been applied or not. It remains unclear whether this effect is due to an increase in the levels of indigenous P. penetrans or other micro-organisms throughout the experiment, or whether dried root material can have a direct influence on nematode activity. The sterilization of soil in microplots in Pakistan eliminated this confusion, but further work is required to establish whether Pp is influenced by other microbial activity in the soil. Carbofuran was not particularly effective in Malawi where there had been a history of carbofuran application, possibly because of microbial degradation (Read, 1983). The use of Pp had no apparent effect on yield at either site in Malawi but the poor overall yields were due largely to the severe incidence of blight (Phytophthora infestans) and it was difficult to maintain healthy plants despite regular fungicide applications.

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LABORATORY SCREENING AS PART OF A STRAIN IMPROVEMENT PROGRAMME FOR *VERTICILLIUM LECANII*, AN IMPORTANT MYCOPATHOGEN OF GLASSHOUSE PESTS

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ABSTRACT

The control of glasshouse pests by the entomopathogenic fungus *Verticillium lecanii* occurs only within a limited range of temperature and humidity. A strain improvement programme could combine complementary characteristics to enhance biological control at low temperatures and humidities. *In vitro* screens examined for responses such as germination and growth at sub-optimal temperatures and water activities are suitable for assessing previously unexamined, wild-type strains and parasexual recombinants. Results are expressed for the two strains of *Verticillium lecanii* of current commercial interest.

INTRODUCTION

Entomopathogenic fungi have considerable potential as biocontrol agents of arthropod pests. They are safe, largely compatible with chemical pesticides and, under appropriate conditions, cause damaging epizootics. However, high humidity and moderate temperatures are required for the germination of their spores, the growth of the mycelium, and the transmission of disease. The critical water activity (aw, equivalent to relative humidity) for spore germination and mycelial growth in most species of entomogenous fungi is approximately 0.93 aw (93% r.h.) (Gillespie & Crawford, 1986). Epizootic development is optimal at 1.00 aw. The most successful activity has been obtained with entomopathogenic fungi against insect pests in environments fulfilling the physiological requirements of the fungi, for example environments suitable for root-feeding and rice pests, spittle bugs, and stem borers (Gillespie, 1988). The activity of entomopathogenic fungi against insect pests might be enhanced under conditions less conducive to fungal infection by improving formulations of the fungi and by strain selection and development (Heale *et al.*, 1989).

Verticillium lecanii, a deuteromycete entomopathogen, is available commercially in Europe for the control of glasshouse pests, in particular aphids, thrips, and the glasshouse whitefly (*Trialeurodes vaporariorum*). The fungus spreads efficiently under suitable conditions (Quinlan, 1988), but control can be sporadic if the humidity falls for large parts of the day (Hall, 1981). Therefore, a strain improvement programme is warranted to enhance characteristics important to the pathogenicity and spread of the fungus under sub-optimal environmental conditions.

Strain improvement of deuteromycete entomopathogens may be achieved by parasexual recombination or by direct gene manipulation (Heale, 1988). There are as yet no primary determinants of pathogenicity encoded by single genes and there do not appear to be prospects of any immediate application of recombinant DNA technology to strain breeding. Parasexual recombinants of *V. lecanii* can be generated by hyphal anastomosis using paired conidial suspensions or by protoplast fusion (Jackson & Heale, 1987). Heterokaryons are obtained by the forced complementation of mutant strains on a selective medium. Conidiospores of heterozygous diploids or recombinant haploids are identified and isolated on fresh selective medium. Recombinant characteristics important to pathogenicity and epizootic potential are assessed *in vitro*, and promising isolates can be examined further in biological assays against target pests.

We have examined *in vitro* screens for some responses of *V. lecanii*, including the growth and germination of the fungus at sub-optimal temperatures and water availability. Such screens are suitable for assessing previously unexamined, wild-type strains and parasexual recombinants. Results are presented in this paper for the two strains of *V. lecanii* of current commercial interest, strain 1.72 ("Vertalec") used in the control of aphids and strain 19.79 ("Mycotal") used in the control of *T. vaporariorum*.

MATERIALS AND METHODS

Media

Fungi were cultured routinely on Czapek-dox complete medium (CM) (Typas & Heale, 1976). Solid media of calculated water activity (Aw medium) was based on a glycerol-water mixture (Dallyn & Fox, 1980) plus agar technical number 3 (Oxoid) (15.0 g/l), glucose (BDH) (2.0 g/l), and yeast extract (Oxoid) (0.1 g/l). Water activities of 0.91-1.00 aw were used.

Assessment of the effects of water activity on the growth of mycelium

Spore suspensions were prepared in sterile distilled water from 7-day spread plates, the concentration of spores was adjusted to 10^7/ml, and 0.1 ml was then spread evenly over a plate of water activity medium (1.0 aw). Plates were incubated at 23 ± 1°C for 48 h. Plugs (1 cm diameter) of mycelium were cut with a cork borer and one plug was placed, lower side uppermost, in the centre of each plate of different aw. Groups of ten plates were sealed in plastic bags and incubated at 23 ± 1°C. The diameters of colonies were measured every 3-4 days for 4 wk. Five plates were used for each strain and each water activity. The experiment was repeated three times.

Assessment of the effects of water activity on the germination of spores of *V. lecanii in vitro*

Spore suspensions were prepared in sterile distilled water from 7-day spread plates and the concentration of spores was adjusted to 10^7/ml. Aliquots (10 µl) of suspension were added to water activity media on each of three designated areas. Plates were left to dry for 15 min and then sealed in plastic bags and incubated at 23 ± 1°C. The germination of

spores was assessed every 4 h. Plates were assessed destructively; at each time period, a drop of lactophenol cotton blue was added to spores to halt germination and the plates were then stored at 5°C before being examined. Two plates were examined for each time period, with no less than 300 spores being counted from several fields of view per plate. Germination was considered as having occurred when the length of the germ tube was equal to the width of the spore. The experiment was repeated three times.

The time (h) for 5%, 50% and 95% of the spores to germinate (Gt5, Gt50, Gt95 respectively) was calculated by probit analysis using the Maximum Likelihood Program (MLP) (Ross, 1980).

Assessment of the effects of temperature on the growth of *V. lecanii* mycelium

The growth of mycelium was measured as above, except that fungi were grown on complete medium at 5, 10 and 23 ± 1°C.

Assessment of the effects of sub-optimal temperatures on the germination of *V. lecanii* spores

The germination of spores was assessed as described previously except that fungal spores were germinated on complete medium at 10 ± 1°C.

RESULTS

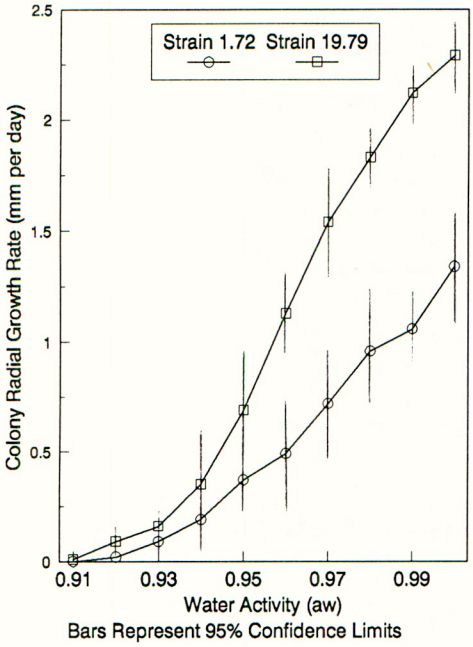
Growth rates of *V. lecanii* strains 1.72 and 19.79 on water activity medium of 0.91 aw to 1.00 aw are shown in Figure 1. Both strains showed maximum growth at 1.00 aw and were severely inhibited below 0.94 aw. The growth rate of strain 19.79 was consistently faster than that of 1.72. The effects of water activity at 23 ± 1°C on Gt5, Gt50, and Gt95 are shown in Figures 2, 3, and 4. Germination was maximal at 1.00 aw and severely restricted below 0.95 aw. Strain 1.72 germinated faster than 19.79 except at 0.93 aw.

Radial growth rates on complete medium (CM) at 5, 10, and 23 ± 1°C are shown in Table 1. Strain 1.72 grew slower than strain 19.79 at all temperatures tested. The germination of both strains on complete medium at 10 ± 1°C (Table 2) was slower than on water activity medium at 23 ± 1°C. Strain 1.72 germinated faster than 19.79, although both strains had a similar lag period.

DISCUSSION

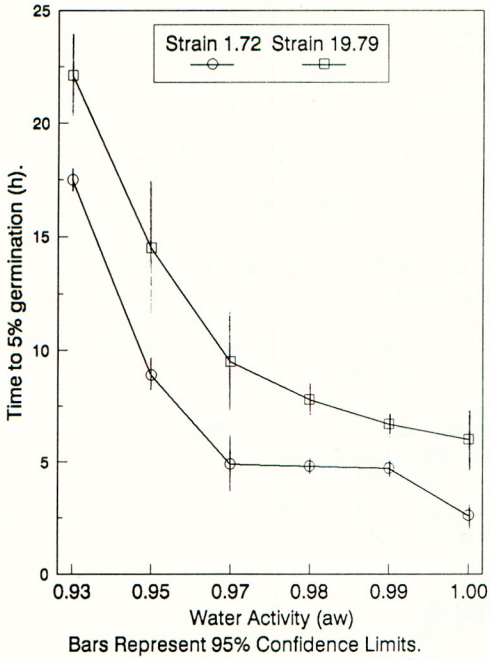
The two commercial strains of *V. lecanii* differed significantly in their response *in vitro* to temperature and water activity. Strain 1.72 germinated quickly but grew more slowly than strain 19.79. Infection of insects by entomopathogenic fungi is initiated by the germination of conidia on the host surface and is followed by fungal penetration of the insects' cuticle. Rapid germination of spores has been correlated with the pathogenicity of *V. lecanii* to the aphid *Macrosiphoniella sanborni* (Jackson *et al.*, 1985). However, low humidity is known to cause a significant reduction in infectivity and disease spread (Milner & Lutton, 1986; Gardner *et al.*, 1984; Drummond *et al.*, 1987). For this reason, it is recommended

Fig.1 The radial growth of V. lecanii mycelium on media of different water activity



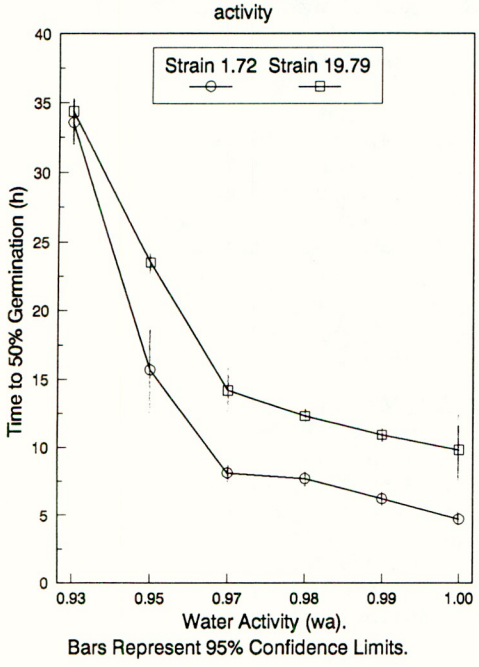
Bars Represent 95% Confidence Limits

Fig.2 The time for 5% of spores of V. lecanii to germinate on media of different water activity



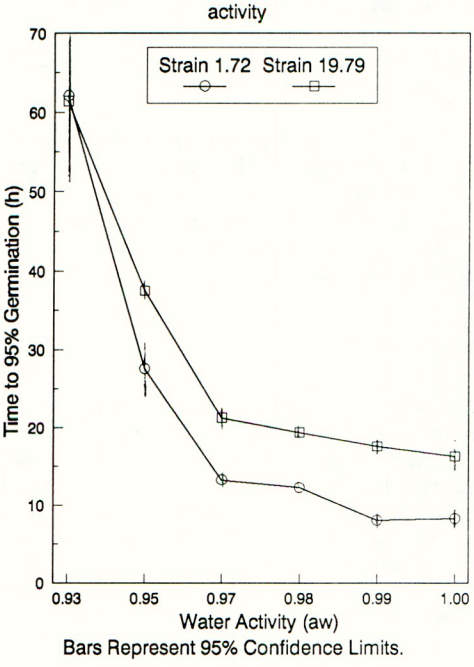
Bars Represent 95% Confidence Limits.

Fig. 3 The time for 50% of spores of V. lecanii to germinate on media of different water activity



Bars Represent 95% Confidence Limits.

Fig. 4 The time for 95% of spores of V. lecanii to germinate on media of different water activity



Bars Represent 95% Confidence Limits.

that spores of *V. lecanii* are applied to glasshouse crops in the late afternoon when humidity is highest (Quinlan, 1988). Application of spores in skimmed milk also permits growth and conidiogenesis on leaf surfaces. Rapid germination and growth at low water activities and temperatures would be an advantageous characteristic. Similarly, fast germination at the time of application, enabling early penetration of the cuticle, would allow infection should the humidity fall subsequently.

The methods used in the experiments reported in this paper are suitable for assessing previously unexamined wild-type strains and parasexual recombinants. Germination rates are measured readily, but the method may be time-consuming when large numbers of recombinants are screened. In this case, a threshold cut-off may be useful, examining plates spread with mono-spored recombinants and discarding those with an insufficiently fast Gt50 value (Heale *et al.*, 1989).

TABLE 1. Radial growth rates of *V. lecanii* on complete medium at different temperatures*

Strain	Radial Growth Rate (mm/day)		
	5°C	10°C	15°C
1.72	0.0 (0.00)	0.7 (0.06)	2.6 (0.05)
19.79	0.1 (0.08)	1.2 (0.05)	3.3 (0.11)

* Figures in brackets refer to the 95% confidence limits of the means of three experiments

TABLE 2. Time for 5% (Gt5), 50% (Gt50) and 95% (Gt95) of spores of *V. lecanii* to germinate on complete medium at 10 ± 1°C*

Strain	(Gt5) (h)	(Gt50) (h)	(Gt95) (h)
1.72	11.0 (2.8)	18.3 (1.1)	30.6 (6.3)
19.79	11.6 (1.0)	27.0 (2.0)	62.6 (6.7)

* Figures in brackets refer to the 95% confidence limits of the means of three experiments

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VERTICILLIUM LECANII AS A MICROBIAL INSECTICIDE AGAINST GLASSHOUSE WHITEFLY

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ABSTRACT

The insect-pathogenic fungus Verticillium lecanii (Deuteromycetes: Moniliales) formulated as a wettable powder (MYCOTAL) can be used in cucumber, tomato and some other protected vegetable and ornamental crops to control whitefly. Repeated sprayings at weekly intervals can reduce whitefly infestations by approximately 90 %, even when relative humidity is as low as 75%. In cucumber, rates of infection of western flower thrips (Frankliniella occidentalis) as high as 60% have been observed. Integration with other biological control agents and fungicide treatments is possible.

INTRODUCTION

V. lecanii is a well-known pathogen of arthropods: it was first described in 1861, and has been collected from numerous species of insects, spiders and mites (Rombach & Gillespie, 1988).
The mode of action is as follows: germinating spores on the cuticle of the insect produce hyphae that penetrate into the body cavity, where the fungus destroys the tissues, thus killing the insect. Later on the fungus grows out through the insect cuticle and sporulates on the outside of the body. These spores can, if spread by splashing water or other mechanical means, spread the infection to other insects.

A specific strain (KV01) of V. lecanii was developed as a microbial insecticide against glasshouse whitefly (Trialeurodes vaporariorum) in the United Kingdom between 1980 and 1985. Trials with this product in the Netherlands in 1984 were not succesful. In 1988 the development of the same strain was resumed by Koppert BV in the Netherlands. Improvements in production and formulation resulted in a wettable powder with a dosage of 10^{10} viable conidiospores per gram, a 50-fold increase in comparison with the former product. Subsequent trials on cucumber and tomatoes in glasshouses proved that the product could have a very good effect on greenhouse whitefly, while the standard operating procedures of the growers could be maintained (Ravensberg et al., 1990). New efficacy trials for registration as a pesticide were successful. Registration in the UK was obtained in 1989. The registration process in the Netherlands and other countries is still underway.

The product is sprayed with standard spraying equipment at a rate of 3 kg per hectare, in two to three thousand litres of water per hectare (10E7 spores per ml). To achieve good control three treatments are recommended since mainly larvae are killed. Temperatures should range from 18 to 30 °C, relative humidity should be above 85 %. The only restrictions are that fungicides should be applied three days

before or three days after V. lecanii applications and that the fungicides tolylfluanid and dichlofluanid should not be used.

EFFECT OF TREATMENTS

In cucumber five trials were done on growers premises. When cucumber is treated three times at weekly intervals the mortality of whitefly larvae usually reaches 85 % two weeks after the first treatment. Four weeks after the first treatment mortality often increases to 95 %, with a range of 70 - 98 % (see fig. 1). In the same cucumber trials F. occidentalis mortality reaches an average of 60 % (see fig. 2).

In tomato (four trials on growers premises) whitefly mortality often reaches 85 % at 2 - 5 weeks after the first of three treatments at weekly intervals (see fig. 3). 80 % control can be obtained after two weeks in glasshouses with low bays (eaves 3 m high) and dense crop structure. At that time, control is only 20 % in taller glasshouses with a more open crop structure, where, although the overall humidity can be the same, the microclimate on the leaf surface is probably quite different. Overall humidity here means the relative humidity of the air in the crop, as a contrast to the relative humidity in the boundary layer of the leaf surface.

INTEGRATION WITH OTHER BIOCONTROL MEASURES

Control of the two-spotted spider mite, Tetranychus urticae, by the predatory mite Phytoseiulus persimilis was not affected by the application of V. lecanii. The whitefly parasite, Encarsia formosa, still parasitised larvae of T. vaporariorum, although the quantity of possible hosts for this parasite was of course reduced by V. lecanii. Negative effects of the fungus were not observed on E. formosa, nor on predatory mites of the genus Amblyseius. This means that V. lecanii can be safely integrated with these biocontrol agents.

DISCUSSION AND CONCLUSIONS

Since it is well known that the relative humidity plays an important role in the infection process of V. lecanii and other entomopathogenic fungi (Quinlan, 1988) the average humidities in various growers nurseries were compared with the percentage mortality of whitefly larvae. There was no relation between whitefly mortality and overall humidity. Since the variation did not appear to be attributed to operator variation or fungicide treatments it was assumed that the microclimate on the leaf was an important cause of variation. This is supported by observations on the effect of crop structure in tomatoes. The overall glasshouse humidity that is measured by the computer is not necessarily correlated with phyllosphere humidity.

It can be concluded that V. lecanii can be used to control whitefly in protected cucumber and tomato crops. It is especially valuable in situations where E. formosa gives, temporarily or locally, insufficient control. It can also be used in the integrated

control programme against F. occidentalis, where every added mortality factor is useful.
Trials in other crops, such as chrysanthemums and poinsettia (against the cotton whitefly Bemisia tabaci) are in progress.

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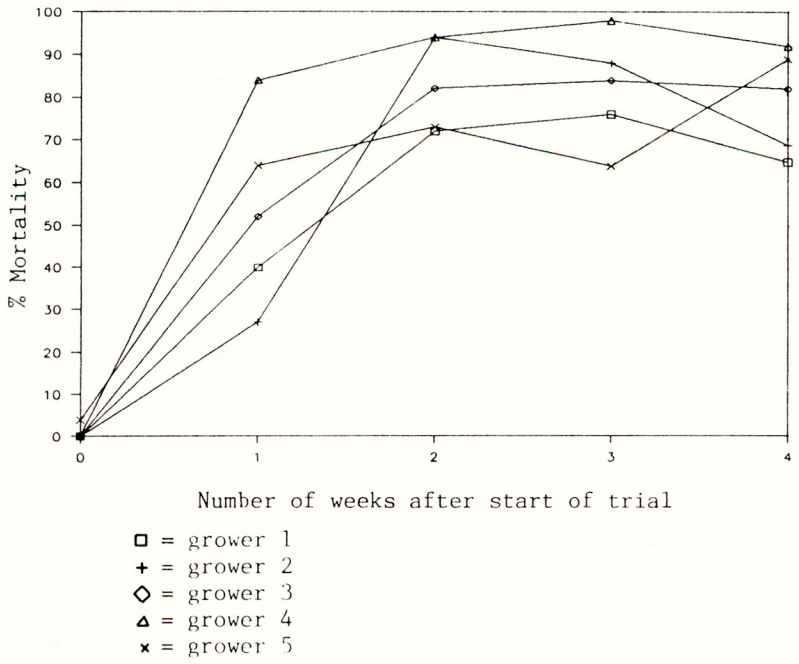

Fig. 1. Mortality of whitefly instars in areas treated with Verticillium lecanii in cucumber; Mean mortality per grower at weekly intervals.

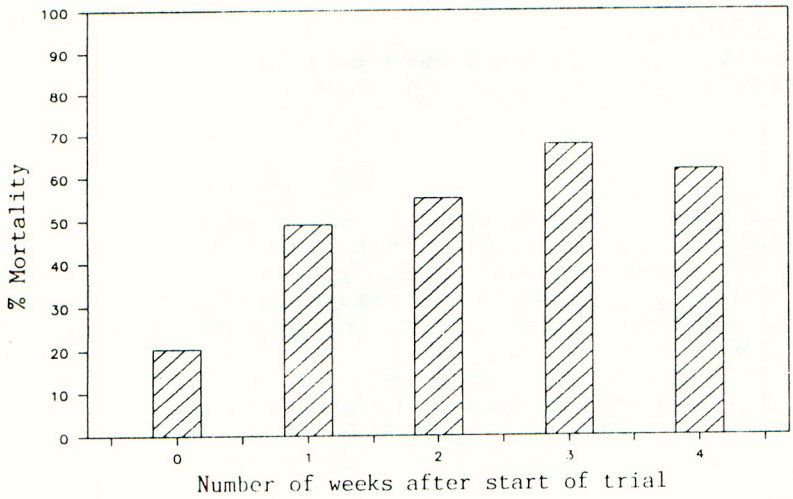

Fig. 2. Mean mortality of Frankliniella occidentalis larvae, pupae and adults in 5 cucumber crops treated with Verticillium lecanii.

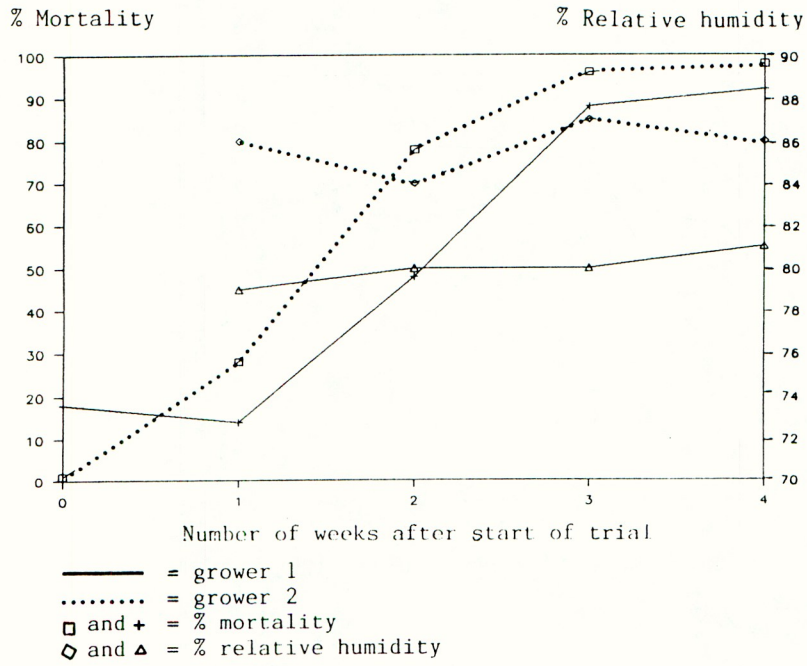

Fig. 3. Mean mortality of whitefly instars in treated areas and mean relative humidity in 2 tomato crops treated with Verticillium lecanii.

A COMPARISON OF NEMATODE-CONTAINING BAITS WITH COMMERCIALLY AVAILABLE INSECTICIDAL BAITS FOR THE CONTROL OF ADULT *MUSCA DOMESTICA* INFESTATIONS OF INTENSIVE ANIMAL UNITS.

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ABSTRACT

Two insecticide baits for adult housefly control, containing azamethiphos or methomyl were compared in laboratory experiments with a bait containing the entomophilic nematode *Steinernema feltiae*. Four strains of houseflies were used; one (S) was susceptible to insecticides and the other (C) had been collected from the field. The two other housefly strains, A and M, had been selectively reared in the laboratory against azamethiphos or methomyl, respectively. After 6 days exposure, azamethiphos was most toxic to all housefly strains, but the bait containing *S. feltiae* was more toxic than methomyl to the selected strains. Modifications to improve the efficacy of the bait containing *S. feltiae* are discussed.

INTRODUCTION

Musca domestica (the common housefly) is a serious pest of animal units in the United Kingdom. However, the number of insecticides available for the control of *M. domestica* is strictly limited to those approved for use under The Control of Pesticides Regulations, 1986. Additionally houseflies are now resistant to a very broad range of insecticides, including organophosphorus, carbamate and pyrethroid compounds (Chapman, 1985); therefore the number of effective insecticidal compounds is severely limited. Azaemthiphos ("Alfacron"; ^{Tm} Ciba Geigy) an organophosphorus compound and methomyl ("Golden Malrin"; ^{Tm} Sandoz), an oxime carbamate, are currently the only two baits approved for housefly control in animal units in the UK.

Renn *et al.* (1986) described the use of entomophilic nematodes of the families Heterorhabditidae and Steinernematidae combined into a bait, which in the laboratory was capable of facilitating parasitism of *M. domestica* and subsequently causing mortality. The author has carried out a number of modifications to this bait system, which includes the use of a British isolate of *Steinernema feltiae*.

The laboratory experiments reported here compare the efficacy of the commerical housefly bait formulations of azamethiphos and methomyl, with the nematode-containing bait in its present form of development. During these experiments the insecticide formulations were applied according to the manufacturers instructions. The numbers of nematodes used and the construction of the nematode bait had been derived by previous experimentation (Renn, unpublished data) to optimise housefly mortality.

MATERIALS AND METHODS

Insect Strains

One-to three-day-old adults of four strains of *M. domestica* were used. The Cooper susceptible strain (C), maintained at the Slough Laboratory since 1979 is susceptible to all insecticides. Strain S was collected from a pig rearing unit, in 1985, where no particular control problems were being experienced. In the laboratory, strain S was found to exhibit low levels of resistance to a number of insecticides, including azamethiphos and methomyl (Nicholas, 1986). The third strain was collected from a pig rearing unit, in 1987, after methomyl ("Golden Malrin"; Tm Sandoz) failed to achieve control. In culture it was subsequently selected through 20 generations against methomyl bait, to produce a strain (M) which survived in significantly high numbers, when exposed to methomyl applied at the recommended commercial application rate (see below). The fourth strain was collected from an intensive egg production unit, in 1988, after azamethiphos ("Alfacron"; Tm Ciba Geigy) had failed to achieve control. This strain was also selected through 20 generations against azamethiphos bait, resulting in a strain (A) which would survive labortory treatments of azamethiphos applied at the manufacturers recommended application rate (see below).

Nematodes

The nematodes were a British isolate of *Steinernema feltiae* cultured *in vitro* and supplied as infective juveniles by Agricultural Genetics Company, MicroBio Division, Cambridge, England. They were counted using 1 ml counting graticules (eelworm counting slides; Hawksley and Sons Ltd., West Sussex, England).

Bait construction

Two discs of horticultural capillary matting (Fibertex SF250, Flowering Plants Ltd., Buckingham, England) were cut to a 9 cm diameter. One disc was used to line the base of a petri-dish. The other was treated with 0.65 mg of the housefly attractant z-9-tricosene ("Flylure"; Denka, Barneveld, Holland) dissolved in 0.5 ml acetone. Once this had dried the attractant-treated disc was placed on top of the first disc within the petri-dish base. To provide a food arrestant, 13 ml unskimmed milk was poured across the surface of the discs. One million nematodes in 2 ml tap water were mixed with 3 ml milk which was also added to the surface of the capillary matting, to complete the bait.

The commercial formulation of azamethiphos bait contains sucrose as a housefly arrestant, but does not contain a pheromone attractant. 885 mg of a 10% azamethiphos formulation was mixed with 0.440 ml water, which was spread evenly across the base of a petri-dish and allowed to dry. This dosage was equivalent to the manufacturer's recommended application rate of 2.5% of the wall and ceiling area of the animal unit being treated with 10,000 mg m^{-2} of azamethiphos. Methomyl bait (225 mg of a 1% formulation), which contains both sucrose and z-9-tricosene, was scattered across the surface of a 9 cm disc of horticultural capillary matting. This dosage was equivalent to the manufacturer's recommended application rate of 100 g m^{-2}.

Assessment of Baits

The four strains of *M. domestica* were exposed to each of the three baits in the following way. Experiments were carried out in aluminium cages (30 x 30 x 30 cm), the two open sides of which were covered with a plastic gauze (540 mesh). A non-toxic alternative food source was provided as 20 ml sucrose solution (10%), absorbed on to cotton wool and placed in a 25 ml disposable beaker. This was changed at 48 h intervals.

One bait of either nematodes, azamethiphos or methomyl was placed centrally on the floor of each cage and approximately 100 houseflies (50♀ and 50♂) were introduced. There were six replicate cages for each treatment and six control cages without a bait. Experiments were carried out at 25°C and 85% rh., with a 14 h light regime of 22 lux intensity.

After introduction of the bait pad (time 0), mortality was assessed at 24 h intervals by removing dead flies from each cage. These were sexed and counted then 25% of the dead flies from the replicates baited with nematodes were dissected to locate parasitising nematodes. Experiments were terminated after six days, when the surviving flies were anaesthetised with CO_2, sexed and counted. Again, 25% of the survivors in the replicates baited with nematodes were dissected.

Statistical analyses

Mortality counts for individual treatments of each strain were compared using Chi-square 2 x 2 contingency tables.

RESULTS

Since male and female houseflies tended to respond to the three bait treatments in a similar manner, only the responses of female houseflies have been considered in detail. However, for each housefly strain certain significant differences occured between the numbers of both sexes responding to the same treatments, at some of the time intervals. These have been indicated in table 1 by suffixes a or b. Although strain A was selected against azamethiphos and strain M against methomyl, houseflies from each of these strains showed tolerance to both of the insecticides. Throughout the majority of these experiments the rate of mortality caused by *S. feltiae* was lower than that of azamethiphos for strains A and M and lower for both azamethiphos and methomyl for strains C and S.

The maximum control mortality was 3.5% of females and 4.3% of males, after 6 days.

Mortality of female *M. domestica*

With the exception of a few survivors of strain S females at day 2, all of the females from strains C and S were killed after exposure to azamethiphos or methomyl (table 1). After exposure to methomyl significantly less females of strain A than strain M had died after 4 and 6 days ($P < 0.01$). At 2, 4 and 6 days azamethiphos proved to be significantly more toxic than methomyl to both strain A and M females ($P < 0.001$), and there was no significant difference in the mortalities of strain A and M females, after exposure to azamethiphos, at each time interval ($P > 0.05$).

Table 1. Percentage mortalities of female *M. domestica* of four strains after exposure to three different baits.

		2 days	4 days	6 days	total number tested
Strain C	*S. feltiae*	31.1[a]	72.6[a]	87.4	309
	methomyl	100.0	100.0	100.0	273
	azamethiphos	100.0	100.0	100.0	303
Strain S	*S. feltiae*	67.7[a]	99.0[a]	100.0	288
	methomyl	98.7	100.0	100.0	311
	azamethiphos	99.0	100.0	100.0	314
Strain A	*S. feltiae*	34.4[a]	71.0[a]	81.5[a]	314
	methomyl	26.9[a]	27.5[a]	32.0[a]	316
	azamethiphos	78.0[b]	93.8[b]	98.7	314
Strain M	*S. feltiae*	48.8[a]	94.0[a]	97.9	285
	methomyl	30.0[a]	40.0	52.3	300
	azamethiphos	72.3[b]	98.2	100.0	285

[a] male response significantly lower ($P < 0.05$)
[b] male response significantly greater ($P < 0.005$)

Steinernema feltiae killed significantly less females of strain C than either of the two insecticides ($P < 0.001$). When strain S females were exposed to *S. feltiae*, at days 2 and 4 significantly less were killed than after exposure to each of the two insecticides ($P < 0.05$). However, by day 6 the three individual treatments had killed all female houseflies from strain S. At each time interval *S. feltiae* had caused a significantly higher mortality of female houseflies of strains A and M than methomyl ($P < 0.05$). However at 2, 4 and 6 days *S. feltiae* killed significantly fewer strain A and M females than azamethiphos ($P < 0.05$). The mortalities of each strain of female houseflies caused by *S. feltiae* displayed some variation. By day 6, 81.5% of strain A females had died as compared with 100% of strain S females, 97.9% of strain M females and 87.4% of strain C. All of these differences were significant ($P < 0.05$).

Dissections of dead houseflies from all four strains, after exposure to *S. feltiae* revealed successful parasitism in 98% of the females and 80% of males examined. The nematodes were distributed evenly throughout the moribund tissues. All houseflies remaining alive after 6 days did not display symptoms of parasitism.

DISCUSSION

Although azamethiphos was the most toxic of the three baits for the the selected strains A and M, the differences in the mortalities caused by the two insecticides were greater than the differences between azamethiphos

and *S. feltiae*. The manufacturer's recommended dosage of azamethiphos was more toxic than that of methomyl. This may have been due to the larger quantity of active ingredient in the azamethiphos formulation ("Alfacron") being recommended by the manufacturers. It is also possible that selecting against one of these insecticides confers cross resistance to the other, since both strains A and M showed tolerance to each of the insecticides; but no attempt was made to measure this here. By contrast, strains C and S both succumbed to azamethiphos and methomyl.

The differences in the mortalities, caused by the two insecticides and *S. feltiae*, at the beginning of each experiment are probably due to differing modes of action. Azamethiphos and methomyl are of the organophosphorus and oxime carbamate groups, respectively. They act directly upon the nervous system of insects (Hassal, 1982) and are therefore rapid in their action. Poinar (1979) reported that steinernematid nematodes can be passively ingested with food. Thus imbibition of the milk, from the bait pad surface, would be one method by which *M. domestica* becomes parasitised by *S. feltiae*. Host finding by *S. feltiae* can also be an active process, in response to chemical and physical cues (Byres and Poinar, 1982). The author has observed *S. feltiae* actively moving across the surface of baits, having emerged from any liquid. This behaviour would increase the likelihood of *S. feltiae* contacting *M. domestica*. Once in contact with *M. domestica*, *S. feltiae* would enter the host's haemocoel via the body apertures (mouth, anus or spiracles) (Kaya, 1985). It would then take 24 h or 48 h for death of *M. domestica* to occur (Kaya, 1985).

During the course of these experiments it was noted that baits containing *S. feltiae* tended to become dry by the end of the experiment. This also meant that the nematodes, which require a moisture-rich environment (Kaya, 1985), tended to be reduced to very low numbers. Renn *et al.* (1986) observed that as few as 10% of the nematodes may remain alive and on the bait pad after 6 days. This may explain why none of the houseflies remaining after six days in the *S. feltiae* treatments had become parasitised. Kaya (1985) described the use of anti-desiccants, to improve the survival of *S. feltiae* in conditions of low moisture. Such additions to the nematode bait may improve the longevity of *S. feltiae*, which in turn may allow greater numbers of houseflies to become parasitised. Alternatively, the remaining houseflies may not have visited the pad. Hecht (1970) stated that surfaces which constrasted well with their surroundings were most attractive to houseflies, so altering the colouration of the *S. feltiae*-containing bait may induce all of the experimental houseflies to visit.

It was encouraging for the future development of nematode baits that the unselected field strain S was killed in equally large numbers by *S. feltiae* as by the two insecticides and that methomyl was not as toxic as *S. feltiae* to the two selected strains A and M. The extra modifications to the *S. feltiae*-containing bait, described above, may result in nematode-induced mortality of houseflies being increased beyond that of azamethiphos.

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COMMERCIALIZATION OF STEINERNEMATID AND HETERORHABDITID ENTOMOPATHOGENIC NEMATODES

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ABSTRACT

Steinernematid and heterorhabditid nematode-based products are commercially available for insect control. Recent developments in mass rearing through liquid fermentation have enabled the nematodes to become economically competitive with chemical insecticides in certain market segments. Substantial improvement in formulation stability and shelf life has been developed by immobilizing or partially desiccating large numbers of nematodes on specific carriers such as clay, polyacrylamide and alginate gels. Large-scale application of nematodes is now feasible due to the ease of mixing and applying these formulations. Standardized protocols and multiple tests are needed to understand the impact of abiotic factors on insect-nematode interactions, thus optimizing nematode efficacy.

INTRODUCTION

Environmental concerns associated with chemical pesticide usage have increased the search for alternative control methods. Entomopathogenic nematodes in the genera, Steinernema and Heterorhabditis, and their associated bacteria Xenorhabdus spp., have received considerable attention recently as biological insecticides, particularly because of their ability to search for and kill hosts rapidly, ease of application, and safety to mammals and plants (Gaugler, 1988). These nematodes have been applied to control insect pests in soil, cryptic and aquatic habitats and on foliage (Kaya, 1985). The most successful and widespread applications of these nematodes have been in soil environments where more than 4×10^{12} nematodes were used in 1988-89 to control insects in various agricultural crops and turfgrass in the United States (Georgis, unpublished data). This paper reports the significant progress achieved since 1985 in production, formulation, field efficacy, and marketing these nematodes.

PRODUCTION

The Bedding (1984) monoxenic culture process has been the production method of choice adopted by a number of universities, decentralized societies such as China, and small commercial operations. The process provides a large surface area to volume ratio by rearing the nematode-bacterium complex on shredded plastic foam soaked with a medium (preferably chicken offal) and placed in flasks or sacks. This process includes a semi-automated harvesting process capable of handling large quantities of foam.

According to Friedman (1990), the economy of the Bedding process is highly dependent upon the consistency of production and the process is sensitive to contamination. In the scale-up model the economy of scale is obtained up to a production level of approximately 10×10^{12} per month. Beyond that point, labor costs remain constant and significant, suggesting the need for a monoxenic liquid fermentation scale production in industrial countries with high labor costs. In the scale-up model beginning at a capacity of 1×10^{12} nematodes per month, production costs using liquid fermentation are less than for other methods, and these costs decrease more rapidly up to a capacity of 50×10^{12} nematodes per month. Over this size multiple plants may be

preferred (Friedman, 1990). Currently steinernematid nematodes are produced consistently and effectively in 15,000 liter with projection to scale up to 60,000 liter. Oxkidney homogenate-yeast extract or a medium containing soy flour, yeast extract, corn oil and egg yolk, have been used to support a yield as high as 100,000 infective juveniles/ml (Friedman, 1990). As a result, the current cost of application of nematode products is ranging between $40.00-$400.00/ha depending on the dosage and the market segment. Optimum aeration and shear sensitivity along with understanding the nematode-bacterium interaction are the most significant issues that need to be considered for production efficiency. These are probably some of the factors affecting the consistency of producing viable heterorhabditid nematodes.

FORMULATION

The ability to store nematodes in product form is critical to commercial success. The formulation and storage of nematodes present unique problems not encountered with a chemical pesticide. Prior to formulation, nematodes are commonly stored under refrigeration in aerated aqueous suspension or on sponges (Table 1). However, the temperature conditions for nematode storage tend to be species specific. Thus, the maintenance of nematode virulence for steinernematids is generally between 5° C and 10° C, and for heterorhabditids is between 10° C and 15° C. Additionally, the oxygen and moisture parameters required by each species must be assessed. Once the requirements for the nematodes are defined, selection of the formulation type, ingredients, packaging size, and storage conditions during each step of product development and distribution can be undertaken.

Substantial improvement in formulation stability has been obtained by immobilizing or partially desiccating the nematodes on specific carriers such as clay, polyacrylamide and alginate gels. Apparently, these carriers reduce nematode metabolism, thus improving their tolerance to temperature extremes. The storage period of such formulations is related to the oxygen and moisture requirements of the nematodes. For example, to achieve 3 months storage at room temperature and 6 months under refrigeration conditions, current products utilize approximately 10 million steinernematid nematodes placed on approximately 12 x 60 cm alginate sheets in 0.5 liter containers. However for heterorhabditid products, 6 million nematodes formulated in a clay can be maintained for 3 months but only at temperatures ranging between 10-15° C. For large scale applications where the recommended dose is high, refrigeration (1-3 months) is suggested for products that utilize one billion steinernematid nematodes placed per 2.3 kg net weight of gel-forming polyacrylamides. In spite of these limitations, nematode products have been introduced successfully in markets were safety and the use of restricted insecticides are an issue. Large-scale application of nematodes is now feasible due to the ease of extracting, mixing and applying these formulations. Anhydrobiotic nematodes may ultimately be a solution to developing a stable formulation for maximum market distribution. Currently, the shelf life of desiccated nematodes falls short of industry's standard of at least 1 year. Research is in progress to characterize and prevent the degradation reactions in desiccated nematodes.

FIELD EFFICACY

In recent years, efforts made to narrow the "efficacy gap" between chemical pesticides and nematodes have been successful in various market segments (Table 2). Research efforts toward adopting a quality control procedure, selecting a suitable target environment and target insects for nematodes, identifying an effective strain and dosage, timing applications properly, and choosing means of application or delivery that increase the probability of successful contact between insect and nematode, have been instrumental in commercializing these entomopathogenic nematodes (Georgis, 1990).

TABLE 1. Storage and shipping requirements for current formulations[a] of steinernematid and heterorhabditid nematodes

Steps in Product Development and Distribution	Storage parameters		
	Method	Condition	Period[b]
Postharvest	Aqueous suspension[c] or on sponge	Refr.[d]	6-12 months
Formulation	Formulated product	Refr.	6-12 months
Shipping	Formulated product	Refr.	1-12 days
		Nonrefr.	1-7 days
Distributor and user	Formulated product	Refr.	1-6 months
		Room temp.	1-3 months

a Immobilized or partially desiccated nematodes in carriers such as alginate, clay or polyacrylamide gel.
b Over 90% nematode viability. Depends on nematode species, concentration, formulation, and package design.
c Aerated
d Refrigeration is 5-10° C for steinernematids and 10-15° C for heterorhabditids

However, multiple trials and standardized protocols between researchers were essential tools to understand the effect of abiotic factors on insect-nematode interactions. Using japanese beetle larvae (Popillia japonica) infesting turfgrass as a model system, Georgis and Gaugler (unpublished data) analyzed 380 treatments from 82 field trials performed between 1984-1988 using a standard protocol. Results showed that most test failure can be explained on the basis of unsuitable nematode species/strains or environmental conditions. Most significantly, data showed that the incidence of nematode failure can be reduced by optimizing application strategies. Abiotic soil factors, for example, can often be manipulated to maximize nematode-insect contact. Adjusting the time of application to avoid low soil temperature and providing irrigation enhanced the performance of nematodes to a level comparable to that of standard insecticides (Table 3). Using a similar strategy the use of nematodes was optimized against other soil-inhabiting insects attacking turf, citrus, cranberry and ornamentals (Table 3). Below are the study conditions and the methodology of the tests.

Depending on the geographical area, mean soil temperatures during the test periods were 21.3°-27.1°C (July), 23.3°-28.6° C (August), 23.9°-29.9° C (September), and 16.2°-20.0° C (October). Irrigation immediately after treatments and at 2-3 day intervals was provided at 1-2 cm level except when adequate precipitation occurred that day. Soil type was sandy or sandy loam in citrus, ornamental and turf (mole cricket) trials, whereas in other trials the soil was characterized as silty clay or silty loam. All treatments were applied between 0600 and 0800 or 1600 and 1800 hrs in a spray volume equivalent to 1500-2300 l/ha. Treatments were applied with conventional hand or ground sprayers (turf, nurseries), sprinkler irrigation or helicopter (cranberry), or microjet irrigation (citrus groves).

Plot sizes ranged between 2.3-29.0 ha (cranberry), 0.2-0.5 ha (citrus groves and nurseries), $9m^2$-$400m^2$ (turf) and 200-4000 potted plants (ornamentals). The plots were non-replicated except for turf tests which were replicated 3-5 times in a randomized complete block design. The number and size of plots were identical for nematode and control treatments.

Treatment effectiveness was determined 3-5 weeks post-treatment (except for black cutworm trials which were evaluated after 7 days) by counting the number of live mature (citrus grove) or immature (other tests) stage insects recovered from soil. The number of samples was as follows: turf; 6-12 soil samples each measuring 20 x 20 x 8 cm/plot. Cranberry; 10-20 soil samples each measuring 20 x 20 x 8 cm/plot. Citrus grove; 100-150 cone adult emergence traps (90 cm diam. base)/ha. Ornamentals and citrus nurseries; 50-90 plants/ha. These counts were compared to untreated control plots and percent population reduction was calculated using Abbott's (1925) formula.

TABLE 2. Current markets for steinernematid and heterorhabditid-based products.

Market segment	Target insects	Countries
Artichoke	Artichoke plume moth (Platyptilia cardiuidactyla)	USA
Citrus	Sugarcane rootstalk borer (Diaprepes abbreviatus)	USA, West Indies
	Blue green weevil (Pachneus litus)	USA
Cranberry & Berries	Black vine weevil (Otiorhynchus sulcatus)	USA,W.Europe,Canada, Australia
	Strawberry root weevil (O. ovatus)	USA, W. Europe, Canada
	Cranberry girdler (Chrysoteuchia topiaria)	USA, Canada
	White grubs (various species)[a,b]	USA, Canada
Turf	Black cutworm (Agrotis ipsilon)	USA, Japan[a], Canada
	Japanese lawn cutworm (Spodeptera depravata)[a]	Japan
	Armyworm (Pseudaletia unipuncta)	USA
	Bluegrass webworm (Parapediasia teterrella)	USA, Japan[a]
	Bluegrass billbug (Sphenophorus parvulus)	USA
	White grubs (various species)[a,c]	USA, Japan, Canada
	Mole crickets (Scapteriscus spp)[a]	USA
Greenhouse/ Nursery Plants	Black vine weevil (O. sulcatus)	USA, W. Europe, Australia, Canada
	Strawberry root weevil (O. ovatus)	USA, W. Europe, Canada
	Fungus gnats (Bradysia spp)	USA, W. Europe, Canada
	White grubs (various species)[d]	USA

a Expected markets in 1991-1993

b Major genera are phyllophaga and Anomala

c Major insects are japanese beetle (Popillia japonica), european chafer (Rhizotrogus majalis), masked chafers (Cyclocephala spp), June beetles (Phyllophaga spp) and soybean beetle (Anomala rufocuprea)

d Major insects are japanese beetle, european chafer and oriental beetle (Anomala orientalis)

TABLE 3. Summary of field efficacy of steinernematid and heterorhabditid nematodes against selected soil-inhabiting insects, 1984-1989[a]

Treatment	Application rate[b]	No. tests	% reduction[c]
	Popillia japonica (japanese beetle, turf)		
H. bacteriophora HP88	2.5×10^9	32	74.8 ± 6.9
S. feltiae #27	2.5×10^9	8	76.6 ± 8.7
Isofenphos	2.50 kg	34	81.4 ± 7.6
	Agrotis ipsilon (black cutworm, turf)		
S. carpocapsae All	2.5×10^9	13	92.9 ± 6.5
chlorpyrifos	1.10 kg	13	99.1 ± 5.3
	Scapteriscus vicinus (tawny mole cricket, turf)		
Steinernema sp #292	2.5×10^9	19	70.3 ± 12.3
Acephate	3.40 kg	22	68.4 ± 11.9
	Diaprepes abbreviatus (sugarcane rootstalk borer, citrus)		
S. carpocapsae All	1.0×10^9 (grove)	12	72.9 ± 9.2
	7.5×10^9 (nursery)	3	96.6 ± 6.4
	Pachnaeus litus (blue green weevil, citrus)		
S. carpocapsae All	1.0×10^9 (grove)	6	77.4 ± 7.3
	7.5×10^9 (nursery)	3	98.0 ± 4.6
	Otiorhynchus sulcatus (black vine weevil, cranberry)		
S. carpocapsae All	7.5×10^9	36	82.3 ± 6.5
	Chrysoteuchia topiaria (Cranberry girdler, cranberry)		
S. carpocapsae All	7.5×10^9	15	87.3 ± 8.7
	P. japonica (japanese beetle, ornamentals)		
S. carpocapsae All	7.5×10^9 (nursery)	6	81.7 ± 10.9
	O. sulcatus (black vine weevil, ornamentals)		
S. carpocapsae All	7.5×10^9 (nursery)	24	88.5 ± 7.6

a Study conditions and methodology detailed in text.

b Expressed as number of infective juveniles for nematode treatments and AI for chemical treatments/ha.

c Each value is the mean ± standard error. Significant differences between treated and control plots using Kruskal-Wallis test (Hollander and Wolfe, 1973).

CONCLUSION

Steinernematid and heterorhabditid-based products have emerged as effective biological soil insecticides, especially in markets where safety and restricted use of insecticides are an issue. Inducing infective stage nematodes into anhydrobiosis may ultimately be a solution to developing a stable, lightweight formulation which is easier to handle and store than present formulations. Identifying and incorporating certain desirable traits (e.g. host-seeking enhancement, UV tolerance, desiccation tolerance) into these nematodes through genetic manipulation may lead to more virulent and pathogenic nematodes. Recent natural isolation of virulent strains/species such as S. scapterisci against mole cricket (Scapteriscus spp), S. kushidai against the japanese beetle (P. japonica), and low temperature pathogenicity strains of S. feltiae (=bibionis) are evidence of the importance of continuing efforts of search for more virulent strains. These efforts along with a better understanding of insect-nematode ecology may enhance the efficacy of nematodes against insects that are currently considered difficult or impractical hosts for nematodes.

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